PATENT ABSTRACTS OF JAPAN

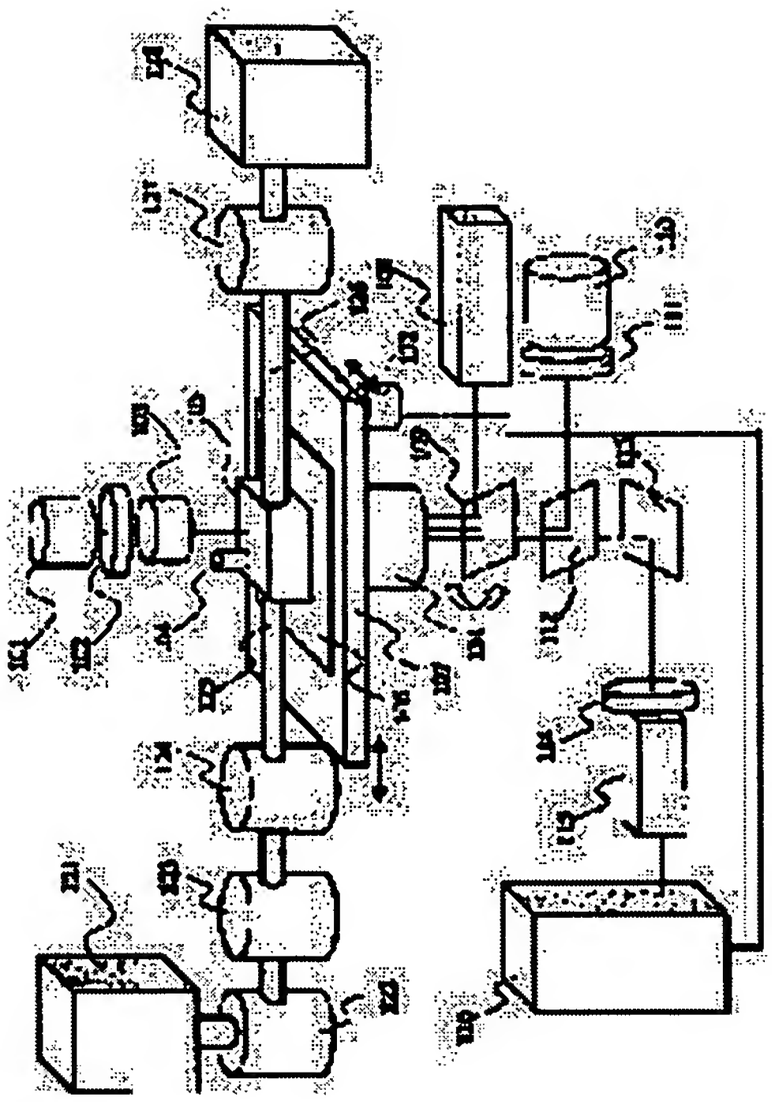
(11)Publication number : **2002-153260**
(43)Date of publication of application : **28.05.2002**

(51)Int.CI. **C12M 1/34**
C12M 1/00
C12M 1/12
C12M 1/26
C12M 1/38
C12M 1/42

(21)Application number : **2000-356827**
(22)Date of filing : **22.11.2000**

(71)Applicant : **JAPAN SCIENCE & TECHNOLOGY CORP**
(72)Inventor : **YASUDA KENJI**
KANEKO KUNIHIKO
YOMO TETSUYA
INOUE IPPEI
WAKAMOTO YUICHI
MORIGUCHI HIROYUKI

(54) DEVICE FOR CULTURING AND OBSERVING ONE CELL WITH MICROSCOPE FOR LONG PERIOD



(57)Abstract:
PROBLEM TO BE SOLVED: To provide a new technical means which enables a work for culturing a cell group originated from a specific one cell, a work for specifying a cell to be interacted in a process for culturing the cell, simultaneously culturing and observing the cell, or a work for culturing a cell in a constant cell concentration, spraying a substance interacting with the cell, such as a signal substance, only on a specific cell in the cell group and then observing a difference between the cell and other cells, and to provide a new means which enables the recovery of only a specific state cell and the subsequent analyses of the gene, expression m-RNA and the like or the biochemical measurement of the cell.
SOLUTION: This new technical means which makes it possible to culture the cell group originated from the specific one cell, characterized by comprising a cell- culturing portion comprising holes formed on a substrate, a semi-permeable membrane for covering the upper surface of the cell-culturing portion, a cell culture container disposed on the semi-permeable membrane and having a culture liquid-exchanging portion, a means for supplying a cell culture solution to the cell culture container, and a micro-optical means capable of observing the cells in the cell culture portion for a long period.

LEGAL STATUS

[Date of request for examination] 13.08.2003

[Date of sending the examiner's decision of rejection]

[Kind of final disposal of application other than the examiner's decision of rejection or application converted registration]

[Date of final disposal for application]

[Patent number]

[Date of registration]

[Number of appeal against examiner's decision of rejection]

[Date of requesting appeal against examiner's decision of rejection]

[Date of extinction of right]

CLAIMS

[Claim(s)]
[Claim 1] 1 cell long-term-culture micro observation equipment characterized by having the cell culture container which has the cell culture section which consists of a hole prepared on the substrate, and the culture medium exchange section which established the top face of the cell culture section in wrap semipermeable membrane and the semipermeable membrane upper part, and providing the supply means of the cell culture liquid to a cell culture container, and the micro optical means which can observe the cell of cell culture circles over a long period of time.
[Claim 2] For 1 micrometers or more, 1mm or less, and the depth, the path of the hole of the cell culture section is 1 cell long-term-culture micro observation equipment of claim 1 characterized by being 100 micrometers or less.
[Claim 3] A container is 1 cell long-term-culture micro observation equipment of claim 1 characterized by being optically made of the transparent quality of the material.
[Claim 4] Semipermeable membrane is 1 cell long-term-culture micro observation equipment of claim 1 characterized by being fixed to the top face of a substrate by association which used avidin and a biotin.
[Claim 5] Semipermeable membrane is 1 cell long-term-culture micro observation equipment of claim 1 characterized by being [with a molecular weight of 10000 or more and a pore size of 0.2 micrometers or less] transparent semipermeable membrane optically.
[Claim 6] The hole of the cell culture section is 1 cell long-term-culture micro observation equipment of claim 1 characterized by being prepared in the top face of or more at least two substrate.
[Claim 7] 1 cell long-term-culture micro observation equipment of claim 6 characterized by the hole of the cell culture section being opened for free passage by the passage established in the substrate top face which can pass a cell in another hole.
[Claim 8] 1 cell long-term-culture micro observation equipment of claim 1 characterized by for the waste fluid discharge means possessing in the cell culture container, being exchanged for the waste fluid in cell culture **** through semipermeable membrane in the culture medium supplied to the culture medium exchange section from a culture medium supply means, and discharging waste fluid by the waste fluid discharge means.
[Claim 9] 1 cell long-term-culture micro observation equipment of claim 1 characterized by arranging the valve for discharging in a container the gas which remained in the container.
[Claim 10] 1 cell long-term-culture micro observation equipment of claim 1 characterized by the means for controlling the temperature of culture medium possessing.
[Claim 11] 1 cell long-term-culture micro observation equipment of claim 1 characterized by catching a cell and providing the means to which it is made to move.
[Claim 12] 1 cell long-term-culture micro observation equipment of claim 11 characterized by catching a cell and providing a photo pincette means as a means to which it is made to move.
[Claim 13] 1 cell long-term-culture micro observation equipment of claim 11 characterized by catching a cell and providing the means using a supersonic wave as a means to which it is made to move.

[Claim 14] 1 cell long-term-culture micro observation equipment of claim 11 characterized by catching a cell and providing the means using electric field as a means to which it is made to move.
[Claim 15] 1 cell long-term-culture micro observation equipment of claim 1 characterized by providing the pipet which sprinkles a reagent in the hole of the cell culture section, and collects reagents.
[Claim 16] 1 cell long-term-culture micro observation equipment of claim 1 characterized by the ability to carry out the fluorescence observation of the cell by putting in a filter on the optical path of a micro optical means.
[Claim 17] 1 cell long-term-culture micro observation equipment of claim 1 characterized by having a means to control the location of a stage, and the focal depth of an objective lens in order to maintain a specific cell in the center of a visual field, a means to acquire image data, a means to recognize the configuration of a specific cell by image data, and.
[Claim 18] 1 cell long-term-culture micro observation equipment of claim 1 characterized by having a means to measure the number of cells in the hole of the cell culture section.
[Claim 19] 1 cell long-term-culture micro observation equipment of claims 1 or 15 characterized by providing the pipet which can penetrate and introduce semipermeable membrane into the hole of the cell culture section, collects the specific cells inside a hole or pours in a reagent and enclosure material.
[Claim 20] The 1 cell long-term-culture micro observation approach characterized by observing a cell over a long period of time using the equipment of claim 1 thru/or either of 19.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] Invention of this application relates to 1 cell long-term-culture micro observation equipment. Invention of this application relates to the approach using the long-term-culture micro observation equipment of a cell and this which can be cultivated per 1 cell in more detail in the area of research of biotechnology which used the microorganism and the cell, carrying out microscope observation of the condition of a specific cell.
[0002]
[Description of the Prior Art] In the field of the conventional biology, medicine, and pharmaceutical sciences, although the response to change of the condition of a cell or the drug of a cell is observed, the average of the value of a cell population has been observed as if it is the property of one cell. However, that with which the cell cycle is aligning in the ensemble of a cell in fact is rare, and protein is discovered with the period from which each cell differed. Although the technique of synchronous culture is developed in order to solve these problems when the difference in the gene of each origin cell before culture may produce the difference in a protein manifestation and the result of the response to a stimulus is actually analyzed, since the origin of the cultivated cell is not from the one same cell at all The fluctuation of the cell reaction mechanism itself was difficult for showing clearly whether to be the fluctuation which originates whether it is a thing originating in the response fluctuation which it has universally in the difference in a cell

(namely, difference in genetic information). moreover, since it was the same and was not what was generally completely cultivated from one cell also about the cell strain, the repeatability of the response to a stimulus was difficult for what swings by the difference in the gene of each cell, or clarifying. Although the stimulus (signal) to a cell is based on the physical contact of what is given with the amount of the signal matter contained in the solution of the cell circumference, a nutrition, and a dissolved gas, and other cells, two kinds have it further again. When a cell was conventionally observed in the area of research of biotechnology, it was observing by taking out temporarily from an incubator a part of cell population cultivated in the large-sized incubator, and setting to a microscope. Or microscope observation was performed, having enclosed the whole microscope with the container of plastics, having managed temperature, and managing carbon dioxide levels and humidity using another small container in it. Much proposals are made as an approach of making solution conditions regularity by exchanging the culture medium which became old, and fresh culture medium, cultivating a cell at this time. For example, by the approach currently indicated by JP,10-191961,A, if it raising and lowers, and a circulating pump operates the level of the culture medium to a base material front face between level higher than the upper limit marginal height of a base material, and level lower than lower limit marginal height and falls in the above-mentioned low, a culture medium will be supplied, and if it goes up to the above-mentioned high level, the nutriture is kept constant according to the device which discharges a culture medium. Moreover, one edge each of the trachea which opens for free passage introductory tubing which introduces a culture medium new in a culture container in JP,8-172956,A into a culture container, the exhaust pipe which discharges the culture medium of a culture container outside, and the gas part and the pump of a culture container was inserted, the filter which prevents invasion of the bacillus into a culture container has been prepared in each duct of said introductory tubing, an exhaust pipe, and a trachea, and it has composition which keeps the nutriture of a cultivation tank constant.

[0003] However, the approach of cultivating, while controlling the solution environment of a cultured cell and the physical contact between cells is not learned in spite of these proposals. Moreover, when cultivating, only one specific cell is chosen and the technique of cultivating the one cell as a cell strain is not known. And when observing a cell, the technique which carries out culture observation is not known, either, specifying the technique which controls the solution environmental condition of a cell and controls the cell concentration in the inside of a container uniformly, or the cell which interacts.

[0004]

[Problem(s) to be Solved by the Invention] When cultivating a cell, in order to start culture from a cell population, in a Prior art, it was not the cell strain which has the same gene completely, so that clearly from the above thing. Moreover, it was difficult to cultivate sorting out specific cells and controlling the interaction or concentration by the Prior art, when cultivating. Furthermore, although the work which keeps a solution environment constant by exchanging the culture medium of a cultivation tank was carried out in the Prior art, it was difficult to change the environment of the specific cell under culture promptly within a cultivation tank, and to observe the response.

[0005] Then, culture observation is carried out [cultivating the cell population which invention of this application cancels the trouble of the conventional technique as above, and originates in one specific cell, or], specifying the cell which carries out an

interaction in the process in which a cell is cultivated, The matter which interacts with cells, such as drugs, such as signal matter, only into the specific cell in the cell population which is cultivating the cell, with cell concentration fixed is sprinkled, and it is making into the technical problem to offer the new technical means which makes it possible to observe the difference of change between the cell and other cells etc. Moreover, invention of this application collects only the cells in a specific condition, and makes it the technical problem to offer the new means which makes it possible to perform analyses, such as a gene of that cell, and Manifestation mRNA, or biochemical measurement.

[0006]

[Means for Solving the Problem] Invention of this application is equipped with the cell-culture container which has the cell-culture section which consists of a hole prepared on the substrate as what solves the above-mentioned technical problem, and the culture-medium exchange section which established the top face of the cell-culture section in wrap semipermeable membrane and the semipermeable-membrane upper part, and offers the 1 cell long-term-culture micro observation equipment characterized by to provide the supply means of the cell-culture liquid to a cell-culture container, and the micro optical means which can observe the cell of cell-culture circles over a long period of time.

[0007] And in invention of this application, it provides [description / of the gestalt on a configuration] about above equipment. for example, with the above 1 cell long-term-culture micro observation equipment of invention of this application A small culture container is arranged on the optical path of a micro observation system. Said interior of a container It consists of the solution exchange **** exchange sections with the coarse eye which is extent which cannot pass the cell culture section which consists of a small hole for cultivating a cell, and the cell which covers the top face so that a cell may not come out from a hole in which culture medium circulates through transparent semipermeable membrane and its top face optically. Moreover, the cell culture section consists of an one or more number of width of face mum to about hundreds of micrometers small hole, and has a means to guide and cultivate the purpose cell in the hole. In the cell culture section, a nutrition and oxygen required for a cell to grow by diffusion from the liquid circulation section are always supplied to a cell from the liquid exchange section, and excrement or secrete has the means removed conversely and a means to observe a cell optically here. Moreover, it has a means to control the class of the number of cells of each hole of the cell culture section, and cell in a hole, by the haulageway made between non-contact capture techniques, such as a photo pincette, and each hole.

[0008] Moreover, with the equipment of invention of this application, it have a means to control the solution temperature inside said container, with temperature control means, such as the Bell Choi component, this invention arrange degassing means, such as a degassing cel and a gas permutation cel, further in liquid sending tubing which send out culture medium to the liquid exchange section from a culture medium reservoir, and it have the means which can control freely the class and concentration of a dissolved gas of culture medium.

[0009] By guiding tips, such as a pipet, to the top face of a specific hole, and sprinkling drugs etc. with the equipment of invention of this application, further again It has a means to have effect of drugs only on the cell in the specific hole which separated semipermeable membrane, and a means to penetrate the above-mentioned semipermeable

membrane with a pipet etc., and to have a means to extract one specific cell from a specific hole, and to introduce an inclusion agent etc. into a specific hole with a pipet etc. similarly.

[0010]

[Embodiment of the Invention] Although invention of this application has the description as above-mentioned, it explains the gestalt of that operation below.

[0011] First, the expression with "1 Cell" as which it is specified in invention of this application that it must clarify is not limited to dealing with only one cell. It is what the cell of two or more individuals may be cultivated in the hole of the cell culture section, and it is that invention of this application is considering as the description to make it possible to control and observe the culture circumstances of a single specific cell etc., even if it is such two or more culture. The convention with "1 Cell" means this.

[0012] moreover, he is the relative convention corresponding [even if it is, are not understood as absolute criteria, and] to the class of each cell convention 5, and I should be understood as a thing with a "long period of time" for which the control and observation of culture circumstances etc. in a long period of time are enabled more compared with the conventional approach.

[0013] Let the above thing be a premise in invention of this application. Drawing 1 shows an example of the basic configuration of the long-term-culture microscope observation equipment of invention of this application. If it explains along with this drawing 1 , the long-term-culture observation equipment of invention of this application cultivated the microorganism and the cell, and is equipped with the culture container 105 for which it enabled it to exchange that culture medium. And adjusting the component of the culture medium sent in this culture container 105, temperature, an ambient atmosphere, a gaseous class, concentration, etc., supply / abandonment system of culture medium which offers culture medium, and the cell in the culture container 105 were observed with time, and it has the micro observation optical system recorded on video, a personal computer, etc.

[0014] If instantiation explanation is given more concretely, the gas exhaust valve 104 for discharging gases, such as air which remained in the container, is formed in the culture container 105 with which a cell is cultivated, and it has the structure where the culture container 105 is filled with culture medium. The magnitude of the base of the culture container 105 is the magnitude suitable for micro observation. Moreover, this culture container 105 is on the stage 107.

[0015] If culture medium supply / abandonment section is explained, solution temperature accommodation will be first carried out at a heater 122, the culture medium supplied from the culture medium feeder 121 which has the function which supplies two or more classes and the culture medium with which concentration differs will be led to the culture container 105 with a tube, and the component of dissolved gases, such as air, will be adjusted in it by the dissolved gas swap device 123. Subsequently, the rate of flow is adjusted with a pump 124, and it is sent to the culture container 105 through a tube 125.

[0016] Another tube 126 is arranged at the culture container 105, and the solution in the culture container 105 is sent to the waste fluid reservoir 128 by suction of a pump 127 through a tube 126. Here, a pump 124 and a pump 127 perform supply and discharge of the culture medium of the culture container 105 by the same rate of flow, when

observing, but after the gas exhaust valve 104 has closed, they can omit a pump 124 or a pump 127. A heater is attached in the waste fluid reservoir 128, it enables it to adjust the temperature of culture medium, and the air in culture medium can also be changed into the condition of having been saturated, by sending air etc. to a culture medium reservoir through a tube with a pump.

[0017] A culture medium reservoir can be connected with the waste fluid reservoir 128 with a tube, and the feeder of culture medium feeder 121 grade can also be made to circulate through culture medium by opened and closing a valve. In this case, a filter is arranged in the middle of a tube, and you may make it remove the excessive component in waste fluid.

[0018] It enables it to irradiate a sample from vertical 2 direction in the optical system in the basic configuration shown in drawing 1 . It is adjusted on specific wavelength by the filter 102, it is condensed with a condensing lens 103, and the light irradiated from the upside light source 101 is irradiated by the culture container 105. The irradiated light is used for observation with an objective lens 131 as the transmitted light. By the mirror 113, the transmitted light image of the culture container 105 interior is guided to a camera 115, and carries out image formation to the light-receiving side of a camera. Therefore, as for the material of the cell culture section substrate 106 which actually cultivates a cell on the culture container 105 and a culture container base, it is desirable that it is a transparent material optically. Specifically, solid-state substrates, such as glass, such as borosilicate glass and quartz glass, resin, such as polystyrene, and plastics or a silicon substrate, are used. Moreover, when using especially a silicon substrate, the light of wavelength with a wavelength of 900nm or more is used for observation. After wavelength selection of the light irradiated from the lower light source 110 is made with a filter 111, it is guided to the objective lens 131 with the dichroic mirror 112. It is used as an excitation light of fluorescence observation of the culture container 105 interior. The fluorescence emitted from the culture container 105 is again observed with an objective lens 131, and can observe only fluorescence and the transmitted light after cutting excitation light with a filter 114 with a camera 115. At this time, by adjusting the combination of filters 102, 111, and 114, only the transmitted light can be observed with a camera 115, only fluorescence can be observed, or a transmitted light image and a fluorescence image can also be observed to coincidence. In the optical path, the device which introduces into an objective lens 131 the laser light which made it generate in the laser light source 108 with the movable dichroic mirror 109 is also equipped. When using this laser as a photo pincette, it is possible to move the focusing location of the laser within the culture container 105 by moving a movable dichroic mirror. moreover , the image data obtained with the camera can drive the motor 132 for stage migration move to an X-Y-Z direction free , in order to control the location of the movable dichroic mirror 109 and the stage with a temperature control function where the culture container 105 appear based on various analysis results , such as a result of having observe the temperature of the thermometry machine which be analyzed by image processing analysis equipment 116 , in addition be attached to the culture container 105 . It is possible to recognize the configuration of a cell by this, or to pursue the cell after recognition, and to double the focus of an image with a specific cell by adjusting distance with an objective lens. [that you make it always located at the core of an image] Or the movable dichroic mirror 109 and the stage 107 with a temperature control function where

the culture container 105 appears are controllable by the period of fixed time amount, or it is at fixed spacing and the motor 132 for stage migration can be driven.
[0019] Drawing 2 illustrates arrangement of the culture container illustrated to drawing 1 . Drawing 3 and drawing 5 illustrate the A-A cross section of this drawing 2 . the cell culture section substrate [in / the tube 203 for the gas exhaust valve 201 and culture medium supply and the tube 204 for discharge of waste fluid are formed in the culture container 202 shown in this drawing 2 like the above, and / in the pars basilaris ossis occipitalis of the culture container 202 / drawing 1] 106 -- the same -- the cell culture section substrate 205 is arranged.
[0020] Although it can consider, for example as glass about the culture container 202, besides glass, it is products made of resin, such as polypropylene and polystyrene, and various kinds of transparent containers can be used optically.
[0021] Moreover, it is also observable with near-infrared light with a wavelength of 900nm or more using solid-state substrates, such as a silicon substrate. The sectional view of drawing 3 is illustrated about the configuration with which the culture container of invention of this application for a cell culture and this were equipped.
[0022] The culture medium sent from the culture medium feeder 121 of said drawing 1 is accumulated in liquid exchange section 301A of the culture container 301 through the tube 302 of drawing 3 . And the fresh culture medium accumulated in this liquid exchange section 301A is exchanged through the culture medium and semipermeable membrane 304 in the cell culture section 306 which became old.
[0023] The cell culture section 306 is constituted by two or more holes established in the substrate 305. The seal of the semipermeable membrane 304 is carried out to the top face of this hole. Therefore, the cell enclosed in the hole 306 has the structure where it cannot come out from this hole, and saprophytic bacteria, such as bacteria, do not enter from the culture medium section.
[0024] The magnitude of this hole needs to be larger than the magnitude of one cell. Therefore, although it is based also on the magnitude of a cell when cultivating a cell, the diameter of opening is 3mm or less, and, generally the depth can set the magnitude to 300 micrometers or less, for example. More preferably, for effective achievement of the expected purpose of invention of this application, it considers as the range whose diameter of opening is 1 micrometer - 1mm, and the thing which is in the range of 10 micrometers - 50 micrometers still more preferably, and the depth may be 100 micrometers or less. Moreover, these diameters of opening and depth are suitably adjusted according to the magnitude of the cell to cultivate, and a class.
[0025] Moreover, considering diffusion of culture medium, also about the height of liquid exchange section 301A of the culture container 301, it is desirable for h to be larger than the depth of a hole. And when performing micro observation and an optical trap using a 100 times as many objective lens as this, a thick thin substrate needs to be used for the thickness of a cell culture section substrate from using an objective lens with high numerical aperture again. For example, when a substrate is boro-silicated glass, it is necessary to use a substrate with a thickness of 0.3mm or less.
[0026] The hole which constitutes the cell culture section 306 may have more than one as aforementioned, and the target cell will be cultivated in this hole. The waste fluid of culture medium will be extracted with a tube 303 from liquid exchange section 301A. Since the depth of the hole of the cell culture section 306 is very shallow, exchange of

culture medium will be performed promptly and culture medium older than a tube 303 will be discharged.
[0027] About semipermeable membrane 304, a cell shall not pass but it shall have the micropore of the magnitude which is extent into which the bacteria of the external world etc. do not go. In invention of this application, semipermeable membrane is 10000 or more molecular weight MW, and, more specifically, its thing with a pore size of 0.2 micrometers or less it is [a thing] optically transparent is desirable.
[0028] Since semipermeable membrane 304 has the pore size which is extent through which a cell does not pass as mentioned above, in saprophytic bacteria entering [the liquid exchange section of the culture container 301] from 301A, a cell does not flow out of the hole of the cell culture section 306 into liquid exchange section 301A.
[0029] A substrate 305 and the culture container 301 are stuck with the bonded seals 307, such as a silicon seal, as illustrated to drawing 3 . Culture medium leaking and coming out from liquid exchange section 301A by this, is prevented. And except the upper part of the hole of the cell culture section 306, the adhesion seal of the semipermeable membrane 304 is carried out to a substrate 305, and it is made for there to be no clearance. This is because a cell cannot be moved between one hole and another hole, when the cell culture section 306 is constituted by two or more holes.
[0030] As a means for adhesion, the approach of of such a substrate 305 and semipermeable membrane 304 which used association with avidin and a biotin, for example is effective. Drawing 4 is the outline sectional view which illustrated this association. The biotin and -(CO)-NH which change - OH radical of semipermeable membrane 401 into -CHO radical partially and by which the amino group was embellished in this between semipermeable membrane 401 and a substrate 402 as semipermeable membrane 401 when glass was used for the substrate 402 of the cell culture section for a cellulose wall - It is made to join together. Thus, a biotin 404 is embellished by semipermeable membrane 401 front face. On the other hand, the amino group can be embellished on a front face by the silane coupling agent in glass substrate 402 front face, and a biotin can be embellished with making it react with a biotin with -CHO radical on substrate 402 front face like semipermeable membrane after that. Avidin 403 is added here and semipermeable membrane 401 is pasted up on the cell culture section substrate 402 by biotin-avidin association.
[0031] Thus, the biotin (404) which carried out joint arrangement joins mutually together through avidin 403, and he is trying to stick to the front faces of semipermeable membrane 401 and a substrate 402 except for the part of the hole of the cell culture section 405. The seal effectiveness excellent in this is realized.
[0032] Drawing 5 is the schematic diagram which illustrated the situation of culture of the cell 503 in the hole of the cell culture section 502 established in the substrate 501. According to culture by invention of this application, even when a 60 times as many objective lens as this is used, for example, the cell 503 in the hole of the cell culture section 502 can be observed with a phase-contrast microscope, a differential interference microscope, and a fluorescence microscope like the usual prepared slide. In addition, semipermeable membrane 504 is also shown in drawing 5 .
[0033] Moreover, in drawing 5 , although the bowl-like hole is illustrated, the configuration may be various kinds, such as a rectangle and a polygon. The hole as the cell culture section can be arranged by the predetermined pattern at equal intervals as two

or more cell culture sections 602 on a substrate 601 as a thing of the magnitude of homogeneity or abbreviation homogeneity like drawing 6 . Moreover, the cell culture sections 702, 703, 704, 705, and 706 as a hole where magnitude differs gradually may be formed on a substrate 701 like drawing 7 . Drawing 8 is the schematic diagram which illustrated the cell 803 in the hole of the cell culture sections 802 and 804 where these magnitude differs, and the situation of culture of 805. At this time, although both the numbers of cells in a hole are 1, the cell concentration which broke the number of cells by the volume of a hole differs. Thus, with the same number of cells, the reaction of the cell in different concentration is observable controlling the volume of a hole.

[0034] And it cannot be overemphasized the arrangement pattern of the hole as the cell culture section, the number of arrangement, and that you may set suitably about the magnitude of a hole or its configuration further. It becomes possible to change cell density by changing the magnitude of the mean free path of the cell made into the purpose object by changing the magnitude (diameter) of the hole of the cell culture section, for example according to invention of this application, or changing the number of the cells for the purpose put into the hole of the same magnitude (diameter). Moreover, the configuration of the hole for a cell culture can be changed and the effect and effectiveness which are given to the cell of the configuration can also be observed.

[0035] Moreover, according to invention of this application, as illustrated, for example to drawing 9 , two or more holes as the cell culture section 902 and this hole may be connected with the substrates 901, such as glass, and the slot 903 as thin passage along which one cell can pass barely may be established in the front face of a substrate 901 at them. Passing speed, performance traverse, etc. of a cell can be measured by forming the slot 903 as this passage. Or a cell is movable to another adjoining cell culture **** through passage using particle prehension means, such as a photo pincette. It is possible to carry out the interaction of separation and the cells of the specification [**** / choose] in a hole for a cell, or to control the number of cells in a hole by using a photo pincette.

[0036] Drawing 10 is the flat-surface schematic diagram which illustrated actuation of such cell migration. The hole as the cell culture section 1002 and the slot 1003 as passage which connects these are established in a substrate 1001, and the cell 1004 is made movable through the slot 1003 in another hole B with photo pincette 1005 means from the hole A of the cell culture section 1002. Photo pincette 1005 means in this case is a means known well until now, and is a means which makes a cell movable by migration of laser focusing light by irradiating laser focusing light at an object cell with the condition of having caught and caught the cell.

[0037] According to the means of such a photo pincette 1005, as illustrated, for example to drawing 11 Make it open for free passage in the slot 1103 as passage, and the hole of the cell culture section 1102 established in the substrate 1101 and the hole of the cell reservoir 1104 are set in structure. the cell of the hole of the cell reservoir 1104 to specification -- a photo pincette -- the hole of the cell culture section 1102 -- **** -- it can come -- reverse -- the cell of specification [the hole of the hole of the cell culture section 1102 to the cell reservoir 1104] -- shift -- or it can also throw away. Or as illustrated to drawing 12 , 1 cell purification culture system can also be constructed on the cell culture section substrate 1201 again. In this case, first, a cell is introduced into the sample induction 1202 from the outside, a slot is moved using prehension means, such as a photo pincette, and one cell in this is guided to the hole 1204 for cell cultures. It has

prevented placing the hole 1203 for cell traps in the middle of this slot, and a cell's swimming from 1202, and advancing into a hole 1204. Next, from the cell population which it was cultivated from one cell and increased in the hole 1204, one cell of a specific condition is taken out again, a slot is similarly moved using a capture means and this is guided to the 2nd hole 1206 for culture. On the way, there is a hole 1205 for cell traps similarly. When the cell cultivated in the hole 1206 changes into the fixed condition of having increased, it is carried through the slot 1207 for cell conveyance in the hole 1208 for cell observation, and observation etc. is made.

[0038] Drawing 13 shows an example of another culture container with which the examples shown by drawing 2 differ. In this example, introduce a pipet 1307 from the exterior, semipermeable membrane 1304 is made to penetrate, and the cells in a hole 1306 are collected alternatively. Therefore, the top face is opened wide, and the culture container 1301 is stretching a mineral oil 1312 on the culture medium 1313 filled in the container, and has prevented mixing of saprophytic bacteria. The amount of the culture medium introduced by the flow of the direction of an arrow head 1311 through a tube 1302 By having become less than the amount of the solution attracted in the direction of an arrow head 1317, and using the oil-level height control section 1315 If this oil-level height becomes lower than the solution outlet 1316, air will be attracted, suction of a solution will stop, the height of an oil level will go up, if an outlet 1316 is taken up, culture medium will be attracted again and the height of an oil level will be kept constant as a result. He is trying for the ripple of an oil level not to affect optical observation in this example by installing the oil-level height control section separately in the culture container 105. A pipet 1307 can also be used in order to attract a cell, but in order to plug up a specific hole and a specific slot, can pour in a bulking agent or can also use it for penetrating semipermeable membrane and introducing a specific cell into the cell culture section here. For example, when introducing a specific sample into the sample induction 1202 of 1 cell purification culture system shown by drawing 12 , it can use. If it moves through a slot with capture means, such as a photo pincette, to the hole by which the seal was carried out by semipermeable membrane before other cells etc. invade from the tear of semipermeable membrane, contamination is satisfactory and it can experiment in the cell introduced by the pipet at this time. Moreover, since the cell of a specific condition is [with this example] recoverable with a pipet 1 with 1 cell unit, gene polymorphism analysis of this one cell, mRNA manifestation analysis, etc. can be performed.

[0039] Drawing 14 shows the example which introduces a reagent in order to apply induction etc. to the cell in the specific hole 1402. The pipet in this case is having dual structure, emits a solution from the inside pipet 1411, and performs suction from the outside pipet 1412. The solution emitted from the inside pipet 1411 by this is distributed only near [the] the outlet, and has the structure where there is no solution in an outer field leakage appearance from the outside pipet 1412 by suction from the outside pipet 1412. Therefore, an operation can be given only to a specific cell by having this pipet near the specific hole.

[0040] In addition, although controlling the concentration of the specific cell in the hole of the cell culture section by migration of the cell by the means of prehension migration, such as a photo pincette as aforementioned of this invention, specification of the cell which interacts, control of an interaction period, etc. are attained, prehension of a cell and the means for migration are not restricted to the above-mentioned photo pincette. For

example, you may be a means using a supersonic wave and may be a means to use electric field.

[0041] One of the examples which introduced two or more electrodes 1501, 1505, and 1508 is shown in the cell culture section substrate 1501 at drawing 15 . From having a specific charge according to the surface state in a solution, a cell can draw near the cell 1511 with an opposite charge to a hole 1503, if positive charge is impressed, for example to an electrode 1502. using this technique -- holes 1503, 1506, and 1509 -- it will be alike, respectively and the cells according to the strength of negative charge will gather. If negative charge is impressed to electrodes 1505 and 1508, it will become impossible moreover, for a cell to move between each hole.

[0042] Moreover, one of the examples which have arranged electrodes 1602 and 1603 to the cell culture section substrate 1601, and have arranged ultrasonic vibrators 1604 and 1605 is shown in drawing 16 . In this example, electric field 1611 and the ultrasonic radiation pressure 1612 are used as non-contact force of operating a cell. Electric field give the external force according to the surface charge which a cell has to a cell, and ultrasonic radiation pressure exerts the external force according to the size and hardness of a cell on a cell. As for the frequency of the supersonic wave used at this time, it is desirable to use the frequency of 1MHz or more in order to control cellular (cavitation) generating by the supersonic wave. In this example, the cell distribution according to the charge and size in which a cell has ultrasonic radiation pressure and electric field concretely by making it act in the different direction which intersects perpendicularly mutually can be developed to two-dimensional. Moreover, although this example performed separation according to the class of cell by combining the external force by electric field, and the external force by the supersonic wave on one substrate, the external force by electric field and the supersonic wave may be used independently, respectively, and you may use for conveyance of a cell. Cell handling which was used with the photo pincette by this is possible.

[0043] And in invention of this application, further, it can have a means to measure the number of cells in the hole as the cell culture section, and semipermeable membrane can be penetrated and introduced into the hole of the cell culture section, and the specific cells inside a hole can be collected or it can also have the pipet which can pour in or collect a reagent and enclosure material in a hole. In addition, it cannot be overemphasized that the thing of various many ways is made possible, without being limited to the above instantiation explanation in any way about the configuration on operation of details.

[0044] For example, in the 1 cell long-term-culture micro observation equipment of this application as above, the following outstanding effectiveness will be acquired, for example.

(1) A specific cell can be isolated and it can observe for a long time.

[0045] (2) The class of culture medium and temperature are freely changeable in the middle of culture.

(3) The volume of the container to cultivate and a configuration can be set up freely.

(4) The number of the cells to cultivate is correctly controllable in the middle of culture.

[0046] (5) Other saprophytic bacteria do not go into the container currently cultivated. While being divided if there is no difference in the growth rate between each generation and the divided die length and it becomes twice as many die length as this in growth

observation of Escherichia coli as illustrated to drawing 17 R> 7 by using the above-mentioned equipment of this application was checked, the magnitude of the hole of the cell culture section, i.e., the request nature to the volume, was checked by growth like drawing 18 . At drawing 18 , it is largr. About 2x10 to 7 ml, it is small. 2x10 to 9 ml is shown.

[0047] Moreover, it was checked that the fission time amount by the generation and the fission time amount by initial number of microorganism have a difference, fission initiation takes time amount like drawing 19 by the fission which is the 1st time, and fission initiation takes time amount rather than the case of two or more cells in being one cell.

[0048] Such a thing will not be made possible without the equipment and the approach of invention this application which enable long term culture in 1 cell level, and micro observation.

[0049]

[Effect of the Invention] Culture observation is carried out [cultivating the cell population which cancels the trouble of the conventional technique and originates in one specific cell by invention of this application as explained in detail above, or], specifying the cell which carries out an interaction in the process in which a cell is cultivated, The matter which interacts with cells, such as drugs, such as signal matter, only into the specific cell in the cell population which cultivates a cell, with cell concentration fixed, and which is cultivated is sprinkled, and the new technical means which makes it possible to observe the difference of change between the cell and other cells etc. is offered. Moreover, invention of this application recovers only the cell in a specific condition, and the new means which makes it possible to perform analyses, such as a gene of that cell and Manifestation mRNA, or biochemical measurement is offered.

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1] It is the mimetic diagram showing an example of the basic configuration of this invention.

[Drawing 2] It is the mimetic diagram showing the equipment configuration of 1 cell culture section shown by drawing 1 .

[Drawing 3] It is the mimetic diagram showing the A-A cross section of 1 cell culture section shown by drawing 2 .

[Drawing 4] It is the mimetic diagram showing an example of the adhesion approach of a substrate and semipermeable membrane.

[Drawing 5] It is the mimetic diagram showing the situation of cell capture of the cell culture section.

[Drawing 6] It is the mimetic diagram showing an example of the structure of the hole on the front face of a substrate.

[Drawing 7] It is the mimetic diagram showing an example of the structure of the hole on the front face of a substrate.

[Drawing 8] It is the mimetic diagram showing the cross-section structure of the hole on the front face of a substrate where the magnitude shown by drawing 7 differs.

[Drawing 9] It is the mimetic diagram showing an example of the structure of the hole on the front face of a substrate.
[Drawing 10] It is a mimetic diagram explaining a means to carry a cell between the holes shown by drawing 9 using the photo pincette.
[Drawing 11] It is the mimetic diagram showing an example of the structure of the hole on the front face of a substrate.
[Drawing 12] It is the mimetic diagram showing an example of the structure of the hole on the front face of a substrate.
[Drawing 13] It is the mimetic diagram showing an example of the equipment configuration of the cell culture section.
[Drawing 14] It is the mimetic diagram showing an example of the equipment configuration of the cell culture section.
[Drawing 15] It is the mimetic diagram showing an example of the structure of the hole on the front face of a substrate.
[Drawing 16] It is the mimetic diagram showing an example of the structure of the hole on the front face of a substrate.
[Drawing 17] It is drawing which illustrated the growth rate between the generations of Escherichia coli, and the observation result of fission die length.
[Drawing 18] It is drawing which illustrated the observation result of the volume dependency about growth of Escherichia coli.
[Drawing 19] It is drawing having shown the fission time amount by the generation, and the observation result of the difference by initial number of microorganism about fission of Escherichia coli.
[Description of Notations]
101 110 Light source
102 111 Filter
103 114 Condensing lens
104 Gas Exhaust Valve
105 Culture Container
106 Cell Culture Section Substrate
107 Stage with Temperature Control Function
108 Laser Light Source
109 Movable Dichroic Mirror
112 Dichroic Mirror
113 Mirror
115 Camera
116 Image-Processing Analysis and Recording Device
121 Culture Medium Feeder
122 Heater
123 Dissolved Gas Swap Device
124 127 Pump
125 126 Tube
128 Waste Fluid Reservoir
131 Objective Lens
132 Motor for Stage Migration
201 Gas Exhaust Valve

202 Culture Container
203 204 Tube
301 Culture Container
301A Liquid exchange section
302 303 Tube
304 Semipermeable Membrane
305 Cell Culture Section Substrate
306 Cell Culture Section
307 Bonded Seal
401 Semipermeable Membrane
402 Cell Culture Section Substrate
403 Avidin
404 Biotin
405 Cell Culture Section
501 Cell Culture Section Substrate
502 Cell Culture Section
503 Cell
504 Semipermeable Membrane
601 Cell Culture Section Substrate
602 Cell Culture Section
701 Cell Culture Section Substrate
702, 703, 704, 705, 706 Cell culture section
801 Cell Culture Section Substrate
802 804 Cell culture section
803 805 Cell
901 Cell Culture Section Substrate
902 Cell Culture Section
903 Slot
1001 Cell Culture Section Substrate
1002 Cell Culture Section
1003 Slot
1004 Cell
1005 Photo Pincette
1101 Cell Culture Section Substrate
1102 Cell Culture Section
1103 Slot
1104 Cell Reservoir
1201 Cell Culture Section Substrate
1202 Sample Induction
1203 1205 Hole for cell traps
1204 1206 Hole for cell cultures
1207 Slot
1208 Hole for Cell Observation
1301 Culture Container
1302 1303 Tube
1304 Semipermeable Membrane

1305 Cell Culture Section Substrate
1306 Hole
1307 Bonded Seal
1308 Pipet
1311, 1314, 1317 Flow of culture medium
1312 Film of Mineral Oil
1313 Culture Medium
1315 Oil-Level Height Control Section
1316 Solution Outlet
1401 Cell Culture Section Substrate
1402 Hole
1403 Cell
1404 Semipermeable Membrane
1411 Solution Emission Pipet Section
1412 Solution Suction Pipet Section
1413 Flow of Pipet Effluent
1414 Flow of Pipet Aspirate
1501 Cell Culture Section Substrate
1502, 1505, 1508 Electrode
1503, 1506, 1509 Hole
1504 1507 Slot
1510 Direction Which Cell Moves
1511 1512 Cell
1601 Cell Culture Section Substrate
1602 1603 Electrode
1604 1605 Ultrasonic vibrator
1611 Sense of Electric Field
1612 Sense of Ultrasonic Radiation Pressure

(19)日本国特許庁（ＪＰ）　(12)**公開特許公報**（Ａ）　(11)特許出願公開番号
特開2002－153260
(P2002－153260A)
(43)公開日　平成14年５月28日(2002.5.28)

(51)Int.Cl.'		識別記号	FI		テーマコード'(参考)
C12M	1/34		C12M	1/34	A　4B029
	1/00			1/00	D
	1/12			1/12	
	1/26			1/26	
	1/38			1/38	Z

審査請求　未請求　請求項の数20　ＯＬ　(全 13 頁)　最終頁に続く

(21)出願番号　特願2000－356327(P2000－356327)

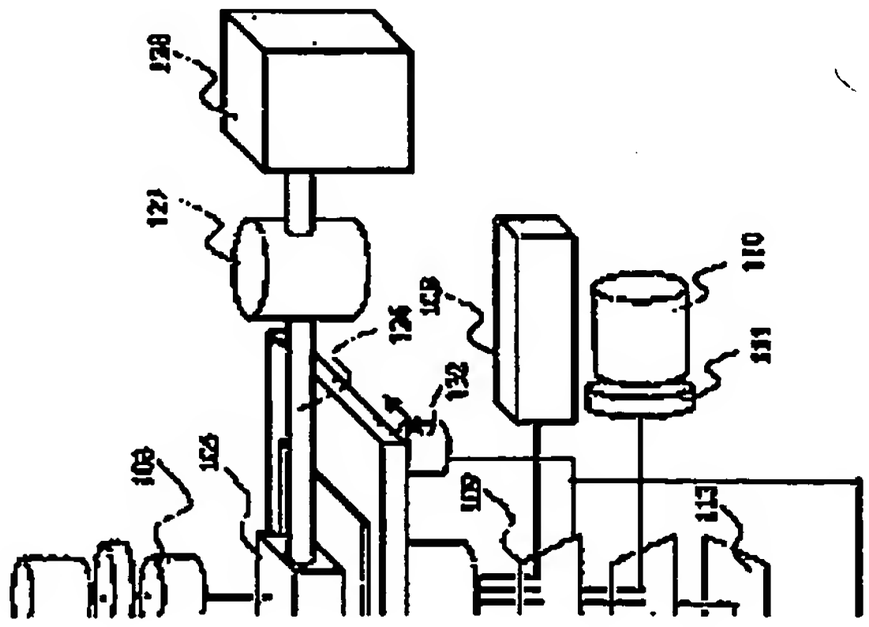
(22)出願日　平成12年11月22日(2000.11.22)

(71)出願人　396020800
科学技術振興事業団
埼玉県川口市本町４丁目１番８号
(72)発明者　安田　賢二
東京都江東区潮見２－８－14－1014
(72)発明者　金子　邦彦
神奈川県藤沢市鵠ケ岡２－７－22
(72)発明者　四方　哲也
大阪府豊中市新千里東町２－４－Ｄ３－106
(74)代理人　100093230
弁理士　西澤　利夫

最終頁に続く

(54)【発明の名称】　一細胞長期培養顕微観察装置

(57)【要約】
【課題】　特定の一細胞に由来する細胞群を培養することや．細胞を培養する過程で相互作用させる細胞を特定しながら培養観察すること．細胞濃度を一定にしたまま細胞を培養する培養している細胞群の中の特定の細胞のみにシグナル物質等の薬剤などの細胞と相互作用する物質を散布し、その細胞と他の細胞との変化の違いを観察すること等を可能とする新しい技術手段を提供する。また．特定の状態にある細胞のみを回収し、その細胞の遺伝子　発現ｍＲＮＡ等の解析．あるいは生化学的測定を



【特許請求の範囲】

【請求項１】　基板上に設けた穴からなる細胞培養部と．細胞培養部の上面を覆う半透膜と．半透膜上部に設けた培養液交換部を有する細胞培養容器を備え．細胞培養容器への細胞培養液の供給手段と．細胞培養部内の細胞を長期観察することのできる顕微光学手段を具備していることを特徴とする一細胞長期培養顕微観察装置。

【請求項２】　細胞培養部の穴の径は１マイクロメートル以上，１ｍｍ以下、深さは１００マイクロメートル以下であることを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項３】　容器は光学的に透明な材質でできていることを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項４】　半透膜は．アビジンおよびビオチンを用いた結合によって基板の上面に固定されていることを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項５】　半透膜は分子量１００００以上．０．２マイクロメートル以下のポアサイズの光学的に透明な半透膜であることを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項６】　細胞培養部の穴は少なくても２つ以上基板の上面に設けられていることを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項７】　細胞培養部の穴が、細胞が通過できる基板上面に設けられた流路によって別の穴に連通されていることを特徴とする請求項６の一細胞長期培養顕微観察装置。

【請求項８】　細胞培養容器には廃液排出手段が具備されており、培養液供給手段より培養液交換部に供給される培養液が半透膜を介して細胞培養部穴内の廃液と交換され．廃液が廃液排出手段により排出されるようにしたことを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項９】　容器には容器内に残留した気体を排出するための弁が配設されていることを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項１０】　培養液の温度を制御するための手段が具備されていることを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項１１】　細胞培養部の穴に試薬を散布する手段を具備することを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項１２】　細胞培養部の穴に試薬を散布し．試薬を回収する手段を具備することを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項１３】　細胞培養部の穴に試薬を散布する手段を具備することを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項１４】　細胞培養部の穴に試薬を散布し．試薬を回収する手段を具備することを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項１５】　細胞培養部の穴に試薬を散布し．試薬を回収するピペットを具備することを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項１６】　顕微光学手段の光路上にフィルターを入れることにより、細胞を蛍光観察できるようにしたことを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項１７】　画像データを取得する手段と．画像データにより特定の細胞の形状を認識する手段と．特定の細胞を視野の中央に維持するためにステージの位置、対物レンズの焦点深さを制御する手段とを有することを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項１８】　細胞培養部の穴の中の細胞数を計測する手段を有することを特徴とする請求項１の一細胞長期培養顕微観察装置。

【請求項１９】　細胞培養部の穴の中に半透膜を貫通して導入でき、穴内部の特定の細胞を回収したり．あるいは．試薬や封入材を注入するピペットを具備することを特徴とする請求項１または１５の一細胞長期培養顕微観察装置。

【請求項２０】　請求項１ないし１９のいずれかの装置を用いて細胞を長期観察することを特徴とする一細胞長期培養顕微観察方法。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】この出願の発明は一細胞長期培養顕微観察装置に関するものである。さらに詳しくは．この出願の発明は、微生物や細胞を用いたバイオテクノロジーの研究分野において、特定の細胞の状態を顕微観察しながら、１細胞単位で培養することのできる．細胞の長期培養顕微観察装置とこれを用いた方法に関するものである。

【０００２】

【従来の技術】従来の生物学、医学．薬学の分野では、細胞の状態の変化、あるいは細胞の薬物等に対する応答を観察するのに．細胞集団の値の平均値をあたかも一細胞の特性であるかの様に観察してきた。しかしながら、実際には細胞の集団の中で細胞周期が同調しているもの

胞株についても．一般には完全に一細胞から培養したものではないため．刺激に対する応答の再現性が細胞各々の遺伝子の違いによってゆらぐものか明らかにするのは難しかった。さらにまた．細胞に対する刺激（シグナル）は、細胞周辺の溶液に含まれるシグナル物質．栄養．溶存気体の量によって与えられるものと、他の細胞との物理的接触によるものの２種類がある。従来．バイオテクノロジーの研究分野において細胞の観察を行う場合は．大型培養器にて培養された細胞群の一部を一時的に培養器から取り出して顕微鏡にセットし、観察を行っていた。あるいは、顕微鏡全体をプラスチックの容器で囲い温度を管理し、その中に小さい別の容器を用い二酸化炭素濃度、及び湿度を管理しつつ．顕微鏡観察を行っていた。このとき、細胞を培養しながら、古くなった培養液と新鮮な培養液を交換することで溶液条件を一定にする方法として多数の提案がなされている。たとえば特開平１０－１９１９６１に開示されている方法では、循環ポンプが、基材表面に対する培地のレベルを基材の上端縁高さより高いレベルと下端縁高さより低いレベルとの間で上げ・下げ操作し．上記低レベルに下がると培地を供給し、上記高レベルに上がると培地を排出する機構によって栄養状態を一定に保っている。また、特開平８－１７２９５６では、培養容器内に．新たな培地を培養容器に導入する導入管と．培養容器の培地を外部に排出する排出管と．培養容器の気体部分とポンプとを連通する気管の各一端を挿入し．前記導入管．排出管及び気管の夫々の管路に培養容器内への菌の侵入を阻止するフィルターを設けており、培養槽の栄養状態を一定に保つ構成になっている。

【０００３】しかし、これらの提案にもかかわらず、培養細胞の溶液環境と、細胞間の物理的接触を制御しながら培養する方法は知られていない。また、培養する場合．特定の一細胞のみを選択し、その一細胞を細胞株として培養する技術は知られていない。そして、細胞を観察する場合に．細胞の溶液環境条件を制御し、かつ、容器中での細胞濃度を一定に制御する技術、あるいは相互作用する細胞を特定しながら培養観察する技術も知られていない。

【０００４】

【発明が解決しようとする課題】以下のことから明らかの従来技術の問題点を解消し、特定の一細胞に由来する細胞群を培養することや．細胞を培養する過程で相互作用させる細胞を特定しながら培養観察すること．細胞濃度を一定にしたまま細胞を培養している細胞群の中の特定の細胞のみにシグナル物質等の薬剤などの細胞と相互作用する物質を散布し、その細胞と他の細胞との変化の違いを観察すること等を可能とする新しい技術手段を提供することを課題としている。また．この出願の発明は．特定の状態にある細胞のみを回収し、その細胞の遺伝子．発現ｍＲＮＡ等の解析、あるいは生化学的測定を行うことを可能とする新しい手段を提供することを課題としている。

【０００６】

【課題を解決するための手段】この出願の発明は．上記の課題を解決するものとして、基板上に設けた穴からなる細胞培養部と．細胞培養部の上面を覆う半透膜と、半透膜上部に設けた培養液交換部を有する細胞培養容器を備え．細胞培養容器への細胞培養液の供給手段と．細胞培養部内の細胞を長期観察することのできる顕微光学手段を具備していることを特徴とする一細胞長期培養顕微観察装置を提供する。

【０００７】そして、上記の装置について、構成上の形態の特徴についてもこの出願の発明において提供している。たとえば．この出願の発明の上記の一細胞長期培養顕微観察装置では、顕微観察系の光路上に小さな培養容器を配置し、前記容器内部は、細胞を培養するための小さな穴からなる細胞培養部と、穴から細胞が出ないようにその上面を被覆する細胞が通過できない程度の目の粗い光学的に透明な半透膜と．その上面は培養液が循環する溶液交換部液交換部で構成される。また、細胞培養部は１つあるいは複数の幅数μｍから数百μｍ程度の小さな穴からなり．その穴に目的細胞を誘導し培養する手段を有している。ここで細胞培養部では液循環部からの拡散により細胞が成長するのに必要な栄養や酸素が液交換部より常に細胞に供給され排泄物あるいは分泌物は逆に取り除かれる手段と、光学的に細胞を観察する手段とを有している。また、光ピンセット等の非接触捕獲技術および各穴の間に作られた連絡路により．細胞培養部の各穴の細胞数、穴の中の細胞の種類を制御する手段を有している。

記半透膜を貫通し、特定の穴から特定の一細胞を抽出する手段を有し．また、同様にピペット等によって特定の穴に封入剤等を導入する手段を有する。

【００１０】

【発明の実施の形態】この出願の発明は、上記のとおりの特徴をもつものであるが．以下に．その実施の形態について説明する。

【００１１】まず、明確にしておかねばならないことは．この出願の発明において規定されている「一細胞」との表現は、一細胞のみを取扱うということに限定されてはいない。細胞培養部の穴においては複数個体の細胞が培養されてよいのであって、この出願の発明が特徴としていることは．このような複数個の培養であっても、単一の特定の細胞の培養経緯等を制御し、かつ観察することを可能としていることにある。「一細胞」との規定はこのことを意味している。

【００１２】また、「長期」との規定いついても．絶対的基準として理解されるものでなく．個々の細胞の種類に応じた相対的な規定であって、しかも、従来の方法に比べてより長期での培養経緯等の制御と観察が可能とされていることとして理解されるべきである。

【００１３】この出願の発明においては、以上のことが前提とされている。図１は．この出願の発明の長期培養顕微鏡観察装置の基本構成の一例を示したものである。この図１に沿って説明すると、この出願の発明の長期培養観察装置は．微生物や細胞を培養し．その培養液を交換できるようにした培養容器１０５を備えている。そして．この培養容器１０５内に送られる培養液の成分や濃度．雰囲気、気体の種類．温度等を調節しながら培養液を提供する培養液の供給・廃棄系と．培養容器１０５内の細胞を経時的に観察し．ビデオやパソコン等に記録する顕微観察光学系を備えている。

【００１４】より具体的に例示説明すると、細胞が培養される培養容器１０５には．容器内に残留した空気等の気体を排出するための気体排出弁１０４が設けられており．培養容器１０５が培養液で満たされる構造になっている。培養容器１０５の底面の大きさは顕微観察に適した大きさである。また、この培養容器１０５は．ステージ１０７の上に乗っている。

【００１５】培養液供給・廃棄部について説明すると．

引により送られる。ここで．ポンプ１２４とポンプ１２７は．観察するときには同じ流速で培養容器１０５の培養液の供給と排出を行うが．気体排出弁１０４が閉じた状態では、ポンプ１２４とポンプ１２７のいずれか一方を省略することができる。廃液溜め１２８にはヒーターが取り付けられて培養液の温度を調節できるようにし、ポンプによりチューブを通して培養液溜めに空気等を送ることにより．培養液中の空気を飽和した状態にすることもできる。

【００１７】廃液溜め１２８には培養液溜めをチューブにより連結し．弁を開閉することによって培養液を培養液供給装置１２１等の供給装置に循環させることもできる。この場合．チューブの途中にフィルターを配設して廃液中の余分な成分を除去するようにしてもよい。

【００１８】図１に示された基本構成での光学系では、上下二方向より試料を照射することができるようにしている。上部の光源１０１より照射された光は、フィルター１０２により特定の波長に調整されコンデンサレンズ１０３によって集光されて．培養容器１０５に照射される。照射された光は、透過光として対物レンズ１３１での観察に用いられる。培養容器１０５内部の透過光像は．ミラー１１３によってカメラ１１５に誘導され、カメラの受光面に結像する。従って、培養容器１０５および培養容器底面で実際に細胞を培養する細胞培養部基板１０６の素材は．光学的に透明な素材であることが望ましい。具体的には、ホウケイ酸ガラス．石英ガラス等のガラスや、ポリスチレン等の樹脂やプラスチック．あるいはシリコン基板等の固体基板を用いる。また．特にシリコン基板を用いる場合は波長９００ｎｍ以上の波長の光を観測に用いる。下部の光源１１０より照射された光はフィルター１１１により波長選択された後に．ダイクロイックミラー１１２によって対物レンズ１３１に誘導されている。培養容器１０５内部の蛍光観察の励起光として用いられる。培養容器１０５から発した蛍光は再度対物レンズ１３１によって観測され．フィルター１１４によって励起光をカットした後の蛍光と透過光のみをカメラ１１５で観察することができる。このとき．フィルター１０２、１１１、１１４の組み合わせを調整することで．透過光のみをカメラ１１５で観察したり．あるいは蛍光のみを観察したり　透過光像と蛍光像を同時に観

１０９や、培養容器１０５が載っている温調機能付ステージの位置を制御するためにＸ－Ｙ－Ｚ方向に自在に移動させるステージ移動用モーター１３２を駆動することができる。これによって細胞の形状を認識したり．認識後にその細胞を追跡し、つねに画像の中心に位置させたり．対物レンズとの距離を調節することで画像のピントを特定の細胞に合わせたりすることが可能である。あるいは．一定時間の周期で可動ダイクロイックミラー１０９や．培養容器１０５が載っている温調機能付ステージ１０７を制御したり、一定間隔でステージ移動用モーター１３２を駆動することが出来る。

【００１９】図２は、図１に例示した培養容器の配置を例示したものであり。図３および図５は、この図２のＡ－Ａ断面を例示したものである。この図２に示した培養容器２０２には．前記と同様に、気体排出弁２０１、培養液供給のためのチューブ２０３と廃液の排出のためのチューブ２０４が設けられて、また培養容器２０２の底部には、図１における細胞培養部基板１０６と同じ、細胞培養部基板２０５が配設されている。

【００２０】培養容器２０２については、たとえばガラス製とすることができるが．ガラス以外にも、ポリプロピレン、ポリスチレン等の樹脂製であって、光学的に透明な各種の容器を用いることができる。

【００２１】また、シリコン基板等の固体基板を用いて波長９００ｎｍ以上の近赤外光で観察することもできる。図３の断面図は、細胞培養のためのこの出願の発明の培養容器とこれに備えられた構成について例示している。

【００２２】前記図１の培養液供給装置１２１から送液された培養液は．図３のチューブ３０２を介して培養容器３０１の液交換部３０１Ａに溜められる。そして、この液交換部３０１Ａに溜められた新鮮な培養液は．細胞培養部３０６内の古くなった培養液と半透膜３０４を介して交換される。

【００２３】細胞培養部３０６は、基板３０５に設けた複数の穴によって構成されている。この穴の上面には半透膜３０４がシールされている。従って穴３０６内に封入された細胞は．この穴から出ることができず．また培養液部からバクテリア等の雑菌が入らないような構造になっている。

【００２５】また培養容器３０１の液交換部３０１Ａの高さについても．培養液の拡散を考えると、穴の深さよりｈが大きいことが望ましい。そしてまた細胞培養部基板の厚みは、１００倍の対物レンズを用いて顕微観察、光トラップを行う場合には．開口数の高い対物レンズを用いることから．肉厚の薄い基板を用いる必要がある。たとえば基板がホウ珪酸ガラスである場合、０．３ｍｍ以下の厚さの基板を用いる必要がある。

【００２６】細胞培養部３０６を構成する穴は．前記のとおり複数あってよく、この穴の中で．目的とする細胞が培養されることになる。培養液の廃液は、液交換部３０１Ａよりチューブ３０３により抜き出されることになる。細胞培養部３０６の穴は、その深さが非常に浅いので．培養液の交換は速やかに行われ．チューブ３０３より古い培養液が排出されることになる。

【００２７】半透膜３０４については．細胞が通りぬけられず、外界のバクテリア等が入らない程度の大きさの微細孔を持つものとする。この出願の発明においては、より具体的には．半透膜は．分子量ＭＷ１００００以上で．０．２μｍ以下のポアサイズの光学的に透明なものであることが好ましい。

【００２８】半透膜３０４は、上記のように、細胞が通りぬけられない程度のポアサイズを有していることから．培養容器３０１の液交換部が３０１Ａから雑菌が入ってくることも．細胞培養部３０６の穴から細胞が液交換部３０１Ａに流れ出してしまうこともない。

【００２９】基板３０５と培養容器３０１とは．たとえば図３に例示したように．シリコンシール等の接着シール３０７によって密着させる。これによって、液交換部３０１Ａから培養液が漏れ出ることが防止される。そして．細胞培養部３０６の穴の上部以外では、基板３０５に対して、半透膜３０４が密着シールされて隙間がないようにする。これは、細胞培養部３０６が複数の穴によって構成される場合、一つの穴と別の穴の間で細胞が移動することができないようにするためである。

【００３０】このような基板３０５と半透膜３０４との密着のための手段としては．たとえばアビジンとビオチンとの結合を利用した方法が有効である。図４はこの結合を例示した概要断面図である。半透膜４０１としてセルロース膜を　細胞培養部の基板４０２にガラスを用い

０１を接着させる。

【００３１】このようにして、細胞培養部４０５の穴の部分を除いて、半透膜４０１と基板４０２の各々の表面に結合配設したビオチン（４０４）が、アビジン４０３を介して相互に結合して密着するようにしている。これに優れたシール効果が実現される。

【００３２】図５は、基板５０１に設けた細胞培養部５０２の穴での細胞５０３の培養の状況を例示した概要図である。この出願の発明による培養によれば、たとえば６０倍の対物レンズを使用した場合でも、通常のプレパラートと同様に、位相差顕微鏡、微分干渉顕微鏡、蛍光顕微鏡で、細胞培養部５０２の穴の中の細胞５０３を観察することができる。なお、図５においては、半透膜５０４も示されている。

【００３３】また、図５では、筒状の穴を例示しているが、その形状は、方形、多角形等の各種であってよい。細胞培養部としての穴は、たとえば図６のように均一ないしは略均一の大きさのものとして、基板６０１上に、複数の細胞培養部６０２として所定の等間隔のパターンで配置させることができる。また、図７のように、基板７０１上に大きさが段階的に異なる穴としての細胞培養部７０２、７０３、７０４、７０５、７０６を設けてもよい。図８は、この大きさの異なる細胞培養部８０２、８０４の穴での細胞８０３、８０５の培養の状況を例示した概要図である。このとき、ともに穴内の細胞数は１であるが細胞数を穴の容積で割った細胞濃度は異なる。このように穴の容積をコントロールすることで同一の細胞数で、異なる濃度での細胞の反応を観察することができる。

【００３４】そして、細胞培養部としての穴の配置パターンや配置数、さらには穴の大きさやその形状については適宜に定めてよいことは言うまでもない。この出願の発明によれば、たとえば細胞培養部の穴の大きさ（直径）を変えることにより目的対象としている細胞の平均自由行程の大きさを変えたり、同じ大きさ（直径）の穴に入れる目的対象細胞の数を変えることにより細胞密度を変化させたりすることが可能となる。また、細胞培養のための穴の形状を変えて、その形状の細胞に与える影響、効果を観察することもできる。

【００３５】また、この出願の発明によれば、たとえば

の中の細胞数を制御することが可能である。

【００３６】図１０は、このような細胞移動の操作を例示した平面概要図である。基板１００１には、細胞培養部１００２としての穴と、これらを連結する流路としての溝１００３を設け、細胞培養部１００２の穴Ａから別の穴Ｂに、光ピンセット１００５手段によって細胞１００４を溝１００３を通して移動可能としている。この場合の光ピンセット１００５手段は、これまでによく知られている手段であって、レーザー集束光を対象細胞に照射することによって細胞を捕捉し、捕捉した状態のまま、レーザー集束光の移動により細胞を移動可能とする手段である。

【００３７】このような光ピンセット１００５の手段によれば、たとえば図１１に例示したように、基板１１０１に設けた細胞培養部１１０２の穴と、細胞溜め１１０４の穴とを流路としての溝１１０３で連通させて構造において、細胞溜め１１０４の穴から特定の細胞を光ピンセットにより細胞培養部１１０２の穴にもってくることができ、逆に細胞培養部１１０２の穴から、細胞溜め１１０４の穴へ特定の細胞を移行ないし捨てることもできる。あるいはまた、図１２に例示したように、細胞培養部基板１２０１上に、１細胞精製培養系を組むこともできる。この場合は、まず、試料導入部１２０２に細胞を外部から導入し、この中の１細胞を光ピンセット等の捕捉手段を用いて溝を移動させ、細胞培養用穴１２０４に誘導する。この溝の途中には細胞トラップ用穴１２０３が設けられており、１２０２から細胞が泳いで穴１２０４に進入するのを防いでいる。次に、穴１２０４で１細胞から培養され増殖した細胞群から、再び特定の状態の細胞１つを取り出し、これを同様に捕獲手段を用いて溝を移動させて、第２の培養用穴１２０６に誘導する。途中には、同様に細胞トラップ用穴１２０５がある。穴１２０６で培養された細胞は、増殖してある一定の状態になったとき、細胞観察用穴１２０８に、細胞運搬用溝１２０７を経て運搬され、観察等がなされる。

【００３８】図１３は、図２で示した実施例とは異なる別の培養容器の一例を示したものである。この例では、外部からピペット１３０７を導入して、半透膜１３０４を貫通させ、穴１３０６中の細胞を選択的に回収する。そのため、培養容器１３０１は上面が開放されており、

別途設置することにより．液面の波紋が光学観察に影響を与えないようにしている。ここでピペット１３０７は．細胞を吸引するために用いることも出来るが．特定の穴や溝を塞ぐために、充填剤を注入したり、特定の細胞を半透膜を貫通して細胞培養部に導入することに用いることもできる。たとえば．図１２で示した１細胞精製培養系の試料導入部１２０２に特定の試料を導入する場合に用いることができる。このときは．ピペットによって導入された細胞は、他の細胞等が半透膜の破れから侵入する前に、光ピンセット等の捕獲手段によって．半透膜でシールされた穴まで溝を通って動かせば、コンタミの問題なく実験することが出来る。また、本実施例でピペット１で１細胞単位で特定の状態の細胞を回収できることから、この１細胞の遺伝子多型解析、ｍＲＮＡ発現解析等も行うことが出来る。

【００３９】図１４は、特定の穴１４０２中の細胞に、誘導等をかけるため試薬を導入する実施例を示している。この場合のピペットは二重構造をしており．内側のピペット１４１１から溶液を放出し．外側のピペット１４１２から吸引を行う。これによって．内側のピペット１４１１から放出された溶液は、その出口近傍にのみ分布し．外側のピペット１４１２からの吸引によって、外側のピペット１４１２から外の領域には溶液が漏れ出ない構造になっている。したがって、このピペットを特定の穴の近傍に持って行くことで、特定の細胞のみに作用を与えることができる。

【００４０】なお、この発明の前記のとおりの光ピンセット等の捕捉移動の手段による細胞の移動で、細胞培養部の穴内での特定細胞の濃度を制御することや．相互作用する細胞の特定、相互作用期間の制御等が可能となるが．細胞の捕捉と移動のための手段は．上記の光ピンセットに限られることはない。たとえば超音波を用いる手段であってもよいし、電場を利用する手段であってもよい。

【００４１】図１５には．細胞培養部基板１５０１に複数の電極１５０１、１５０５、１５０８を導入した実施例の一つを示している。細胞は溶液中でその表面状態に応じて、特異的な電荷を持つことから．たとえば電極１５０２に正の電荷を印加すると、反対の電荷を持つ細胞１５１１を穴１５０３まで引き寄せることができる。この

胞の持つ表面電荷に応じた外力を細胞に与え、また、超音波輻射圧は細胞のサイズおよび硬さに応じた外力を細胞に及ぼす。このとき用いる超音波の振動数は超音波による気泡（キャビテーション）発生を抑制するため１ＭＨｚ以上の周波数を用いることが望ましい。この実施例では．具体的に．超音波輻射圧と電場を互いに直交する異なる方向に作用させることで、細胞の持つ電荷とサイズに応じた細胞分布を２次元に展開することができる。また．この例では電界による外力と超音波による外力を１つの基板上で組み合わせることで細胞の種類に応じた分離を行ったが．電界、超音波による外力をそれぞれ単独で用いて細胞の選別に用いても良い。これによって光ピンセットで用いたような細胞ハンドリングが可能である。

【００４３】そして、この出願の発明においては．細胞培養部としての穴中の細胞数を計測する手段を備えることができ、さらには、細胞培養部の穴に、半透膜を貫通して導入でき．穴内部の特定の細胞を回収したり．あるいは．穴内に試薬や封入材を注入もしくは回収することのできるピペットを備えることもできる。その他細部の実施上の形状については以上の例示説明に何ら限定されることなしに．各種各様のものが可能とされることは言うまでもない。

【００４４】たとえば以上のとおりのこの出願の一細胞長期培養顕微観察装置においては、たとえば次のような優れた効果が得られることになる。

（１）特定の細胞を隔離し．長時間観察することができる。

【００４５】（２）培養液の種類、温度を培養途中に自由に変えることができる。

（３）培養する容器の容積．形状を自由に設定することができる。

（４）培養する細胞の数を培養途中に正確に制御することができる。

【００４６】（５）培養している容器に他の雑菌は入ってこない。

この出願の上記装置を用いることにより、たとえば図１７に例示したように、大腸菌の成長観察においては、各世代間での成長速度、分裂する長さに差はなく．２倍の長さになると分裂することが確認される一方で　図１８

法とによってはじめて可能とされるのである。

【００４９】

【発明の効果】以上詳しく説明したとおり、この出願の発明によって、従来技術の問題点を解消し、特定の一細胞に由来する細胞群を培養することや、細胞を培養する過程で相互作用させる細胞を特定しながら培養観察すること、細胞濃度を一定にしたまま細胞を培養する培養している細胞群の中の特定の細胞のみにシグナル物質等の薬剤などの細胞と相互作用する物質を散布し、その細胞と他の細胞との変化の違いを観察することなどを可能とする新しい技術手段が提供される。また、この出願の発明により、特定の状態にある細胞のみを回収し、その細胞の遺伝子、発現ｍＲＮＡ等の解析、あるいは生化学的測定を行うことを可能とする新しい手段が提供される。

【図面の簡単な説明】

【図１】この発明の基本構成の一例を示す模式図である。

【図２】図１で示した１細胞培養部の装置構成を示す模式図である。

【図３】図２で示した１細胞培養部のＡ－Ａ断面を示す模式図である。

【図４】基板と半透膜の接着方法の一例を示す模式図である。

【図５】細胞培養部の細胞捕獲の様子を示す模式図である。

【図６】基板表面の穴の構造の一例を示す模式図である。

【図７】基板表面の穴の構造の一例を示す模式図である。

【図８】図７で示した大きさの異なる基板表面の穴の断面構造を示す模式図である。

【図９】基板表面の穴の構造の一例を示す模式図である。

【図１０】光ピンセットを用いて図９で示した穴の間で細胞を運搬する手段を説明する模式図である。

【図１１】基板表面の穴の構造の一例を示す模式図である。

【図１２】基板表面の穴の構造の一例を示す模式図である。

【図１３】細胞培養部の装置構成の一例を示す模式図で

ある。

【図１９】大腸菌の分裂について、世代による分裂時間と、初期菌数による差の観察結果を示した図である。

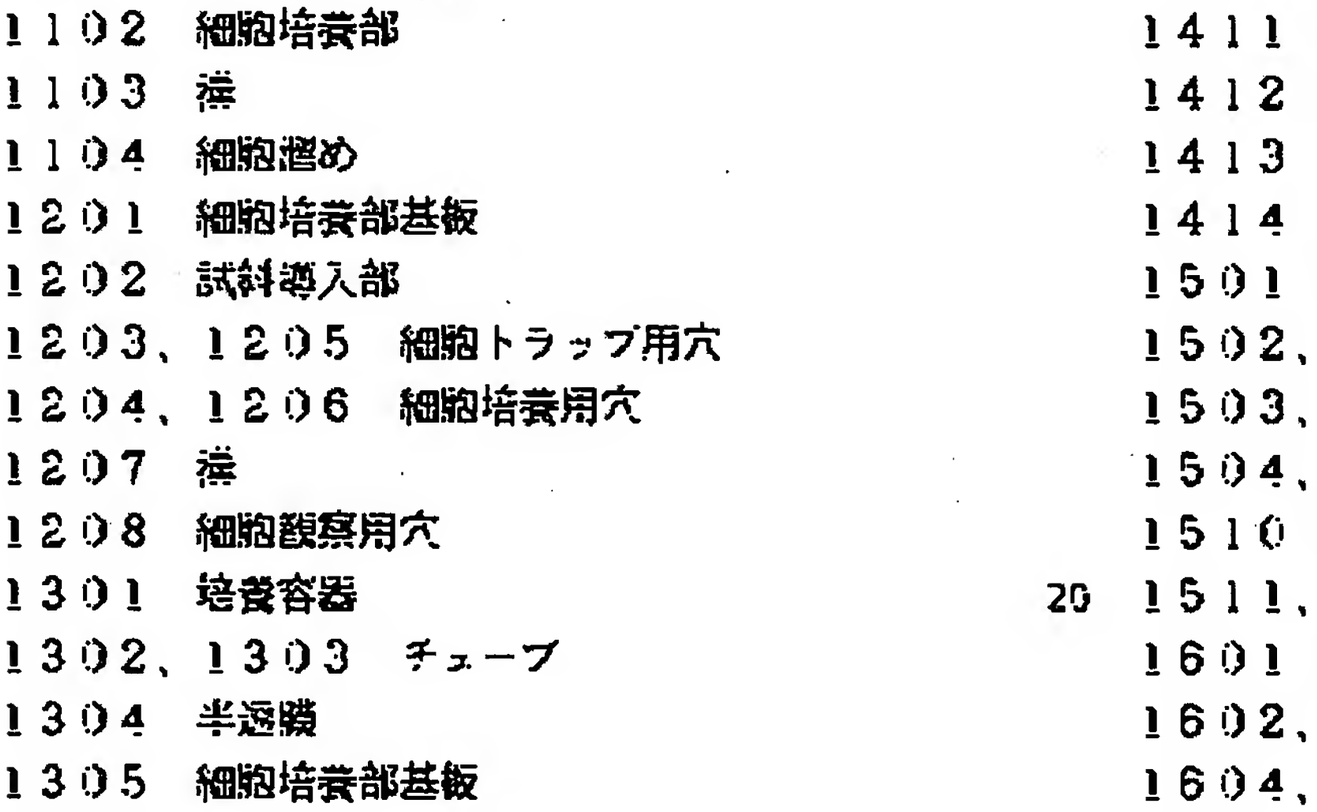
【符号の説明】

１０１、１１０　光源
１０２、１１１　フィルター
１０３、１１４　コンデンサレンズ
１０４　気体排出弁
１０５　培養容器
１０６　細胞培養部基板
１０７　温調機能付ステージ
１０８　レーザー光源
１０９　可動ダイクロイックミラー
１１２　ダイクロイックミラー
１１３　ミラー
１１５　カメラ
１１６　画像処理解析・記録装置
１２１　培養液供給装置
１２２　ヒーター
１２３　溶存気体交換装置
１２４、１２７　ポンプ
１２５、１２６　チューブ
１２８　廃液溜め
１３１　対物レンズ
１３２　ステージ移動用モーター
２０１　気体排出弁
２０２　培養容器
２０３、２０４　チューブ
３０１　培養容器
３０１Ａ　液交換部
３０２、３０３　チューブ
３０４　半透膜
３０５　細胞培養部基板
３０６　細胞培養部
３０７　接着シール
４０１　半透膜
４０２　細胞培養部基板
４０３　アビジン
４０４　ビオチン
４０５　細胞培養部

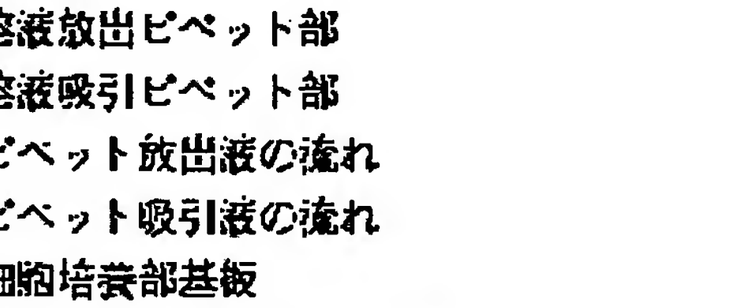
８０３、８０５　細胞
９０１　細胞培養部基板
９０２　細胞培養部
９０３　溝
１００１　細胞培養部基板
１００２　細胞培養部
１００３　溝
１００４　細胞
１００５　光ピンセット
１１０１　細胞培養部基板
１１０２　細胞培養部
１１０３　溝
１１０４　細胞潰め
１２０１　細胞培養部基板
１２０２　試料導入部
１２０３、１２０５　細胞トラップ用穴
１２０４、１２０６　細胞培養用穴
１２０７　溝
１２０８　細胞観察用穴
１３０１　培養容器
１３０２、１３０３　チューブ
１３０４　半透膜
１３０５　細胞培養部基板
１３０６　穴
１３０７　接着シール

＊１３０８　ピペット
１３１１、１３１４、１３１７　培養液の流れ
１３１２　ミネラルオイルの膜
１３１３　培養液
１３１５　液面高さ調節部
１３１６　溶液出口
１４０１　細胞培養部基板
１４０２　穴
１４０３　細胞
１４０４　半透膜
１４１１　溶液放出ピペット部
１４１２　溶液吸引ピペット部
１４１３　ピペット放出液の流れ
１４１４　ピペット吸引液の流れ
１５０１　細胞培養部基板
１５０２、１５０５、１５０８　電極
１５０３、１５０６、１５０９　穴
１５０４、１５０７　溝
１５１０　細胞の動く方向
１５１１、１５１２　細胞
１６０１　細胞培養部基板
１６０２、１６０３　電極
１６０４、１６０５　超音波振動子
１６１１　電場の向き
＊　１６１２　超音波輻射圧の向き

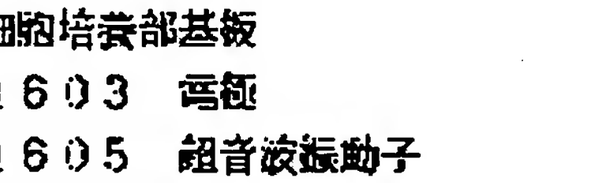
【図１】



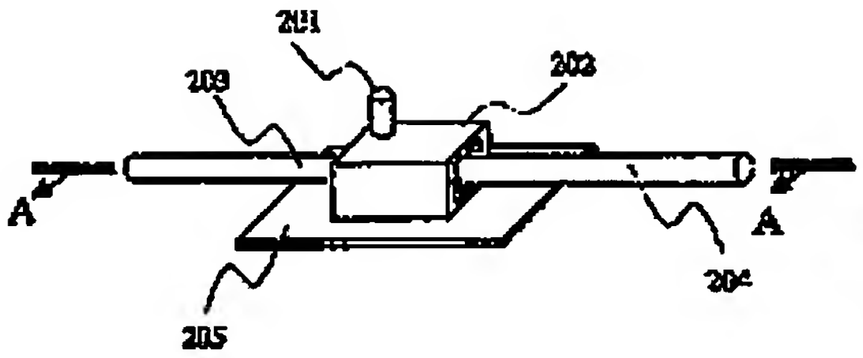
【図６】



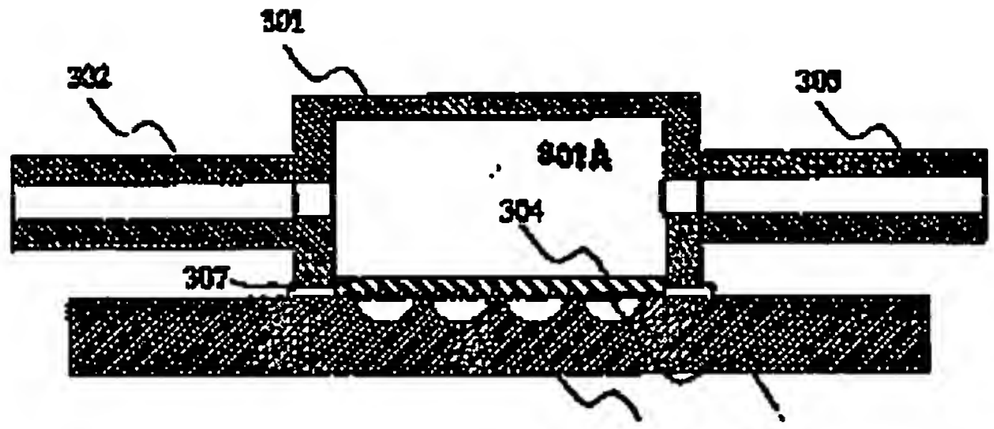
【図７】



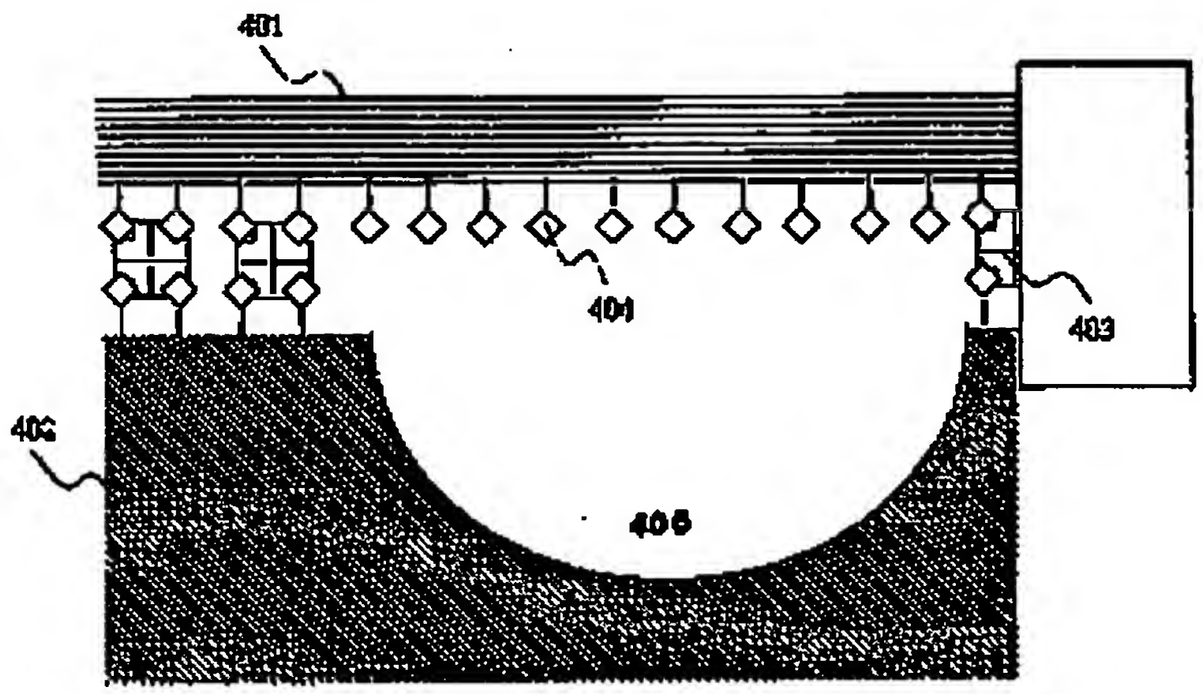
【図２】



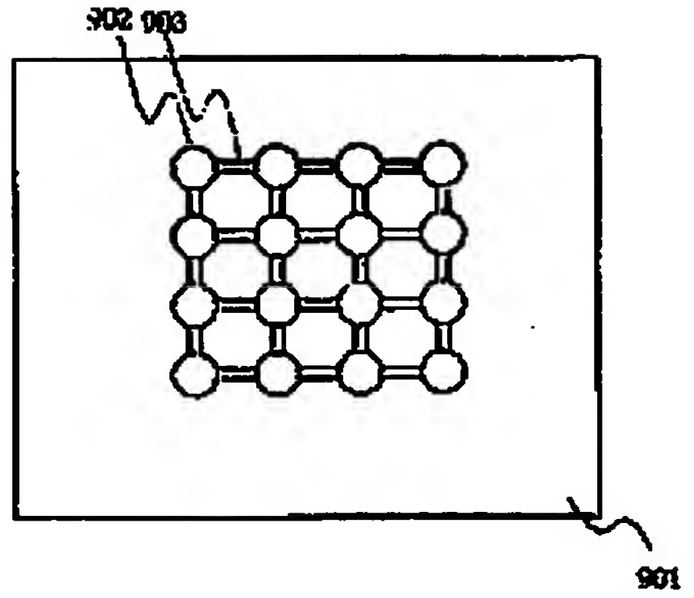
【図３】



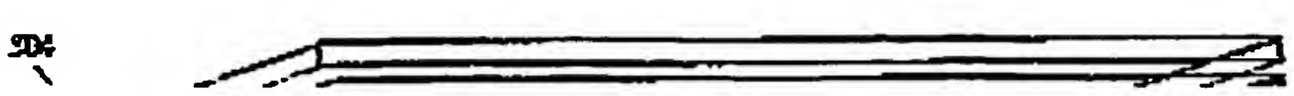
【図４】



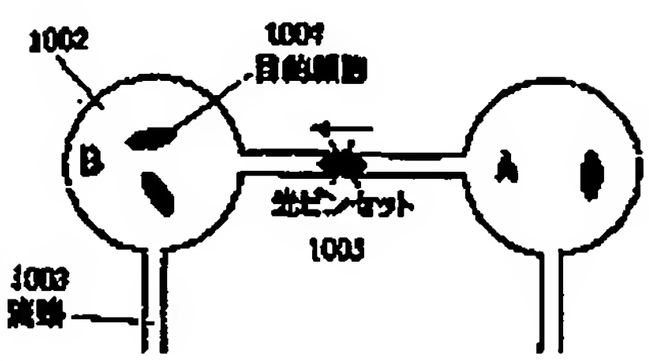
【図９】



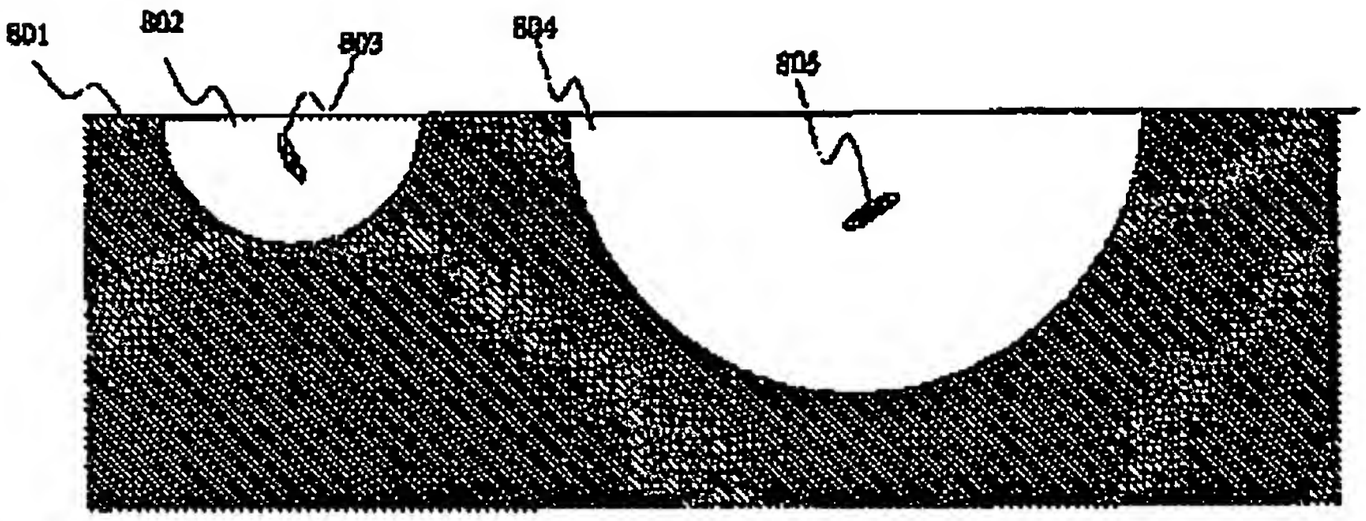
【図５】



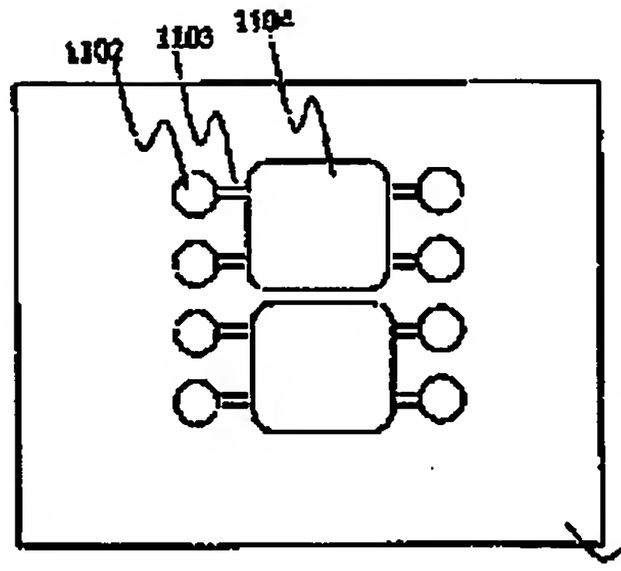
【図１０】



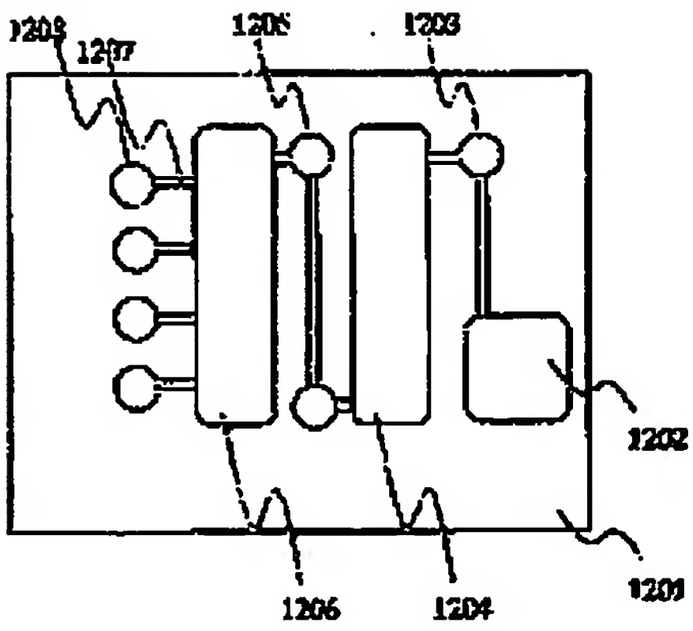
【図８】



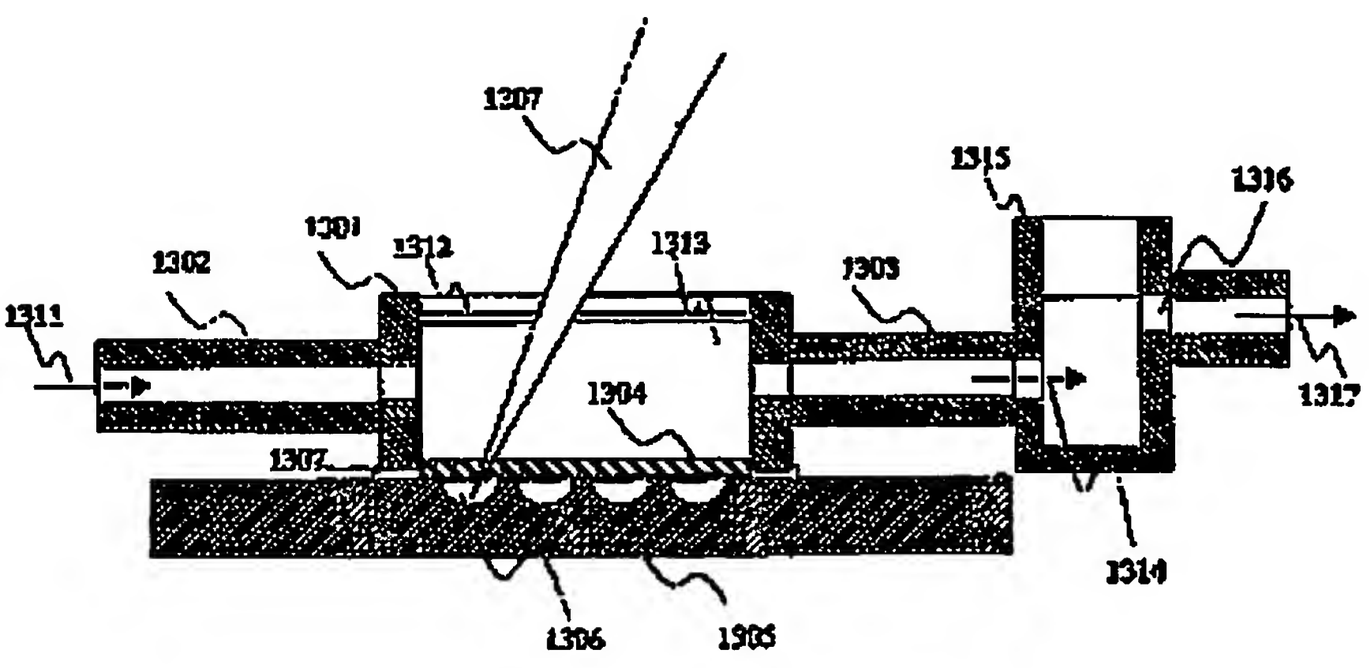
【図１１】



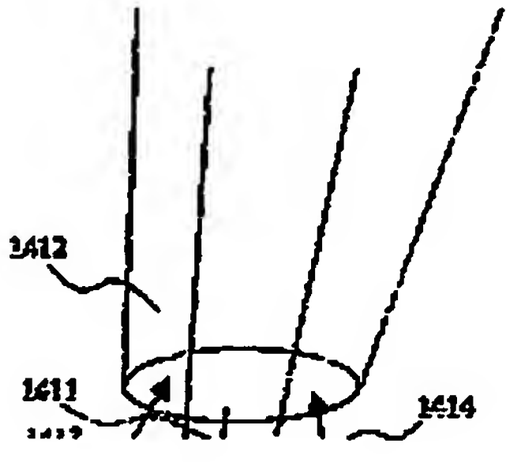
【図１２】



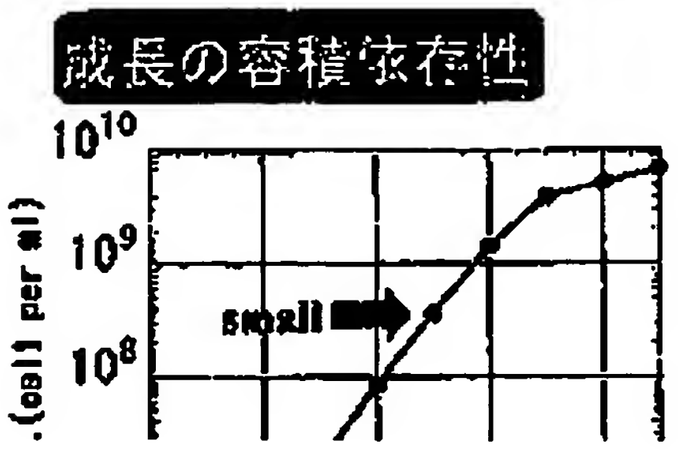
【図１３】



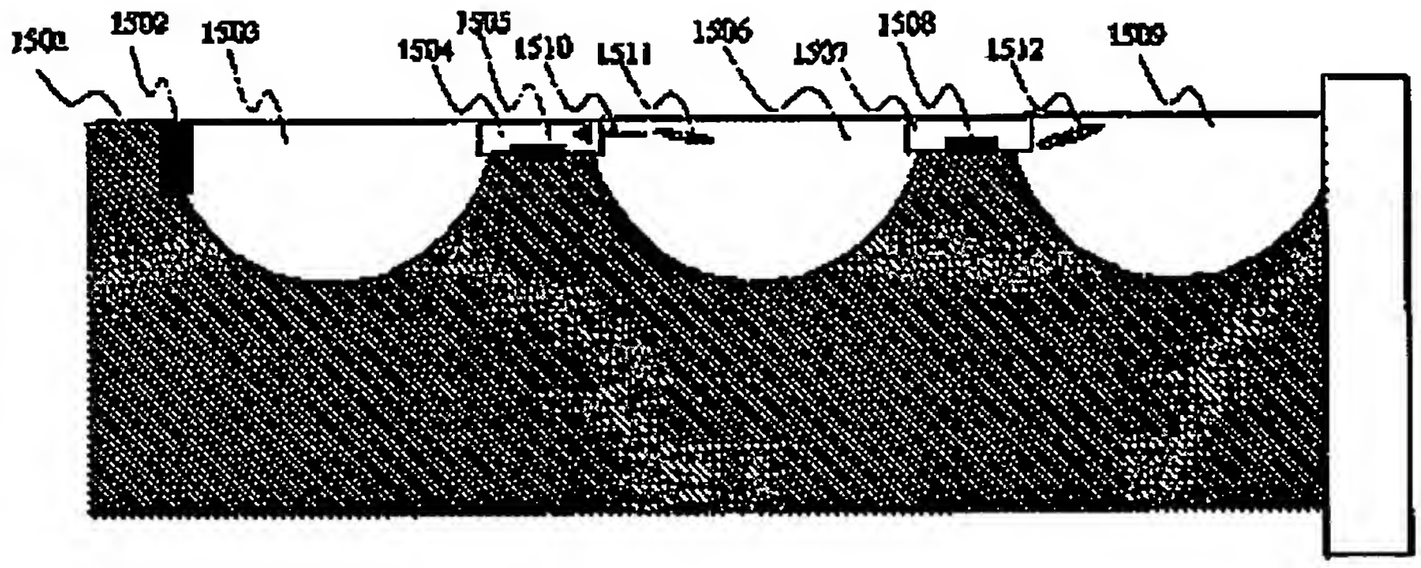
【図１４】



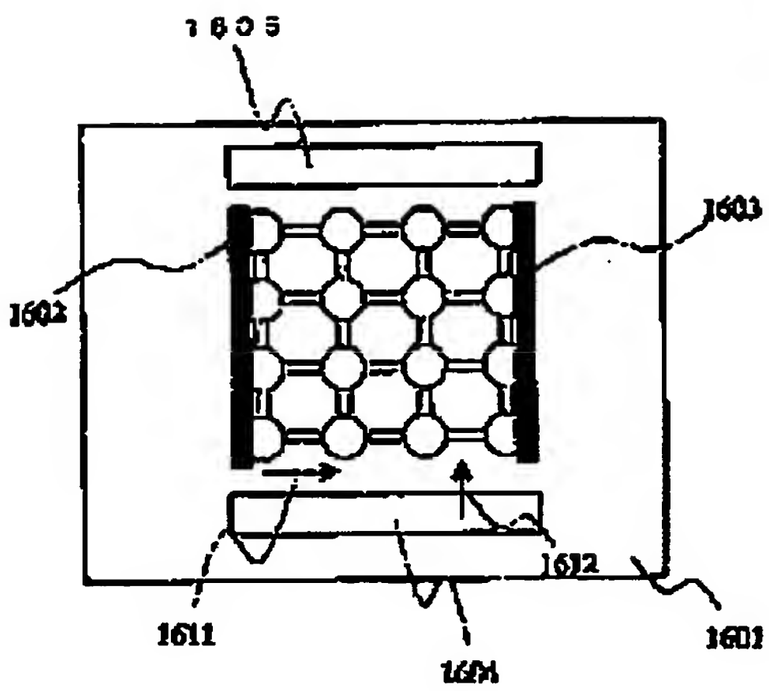
【図１８】



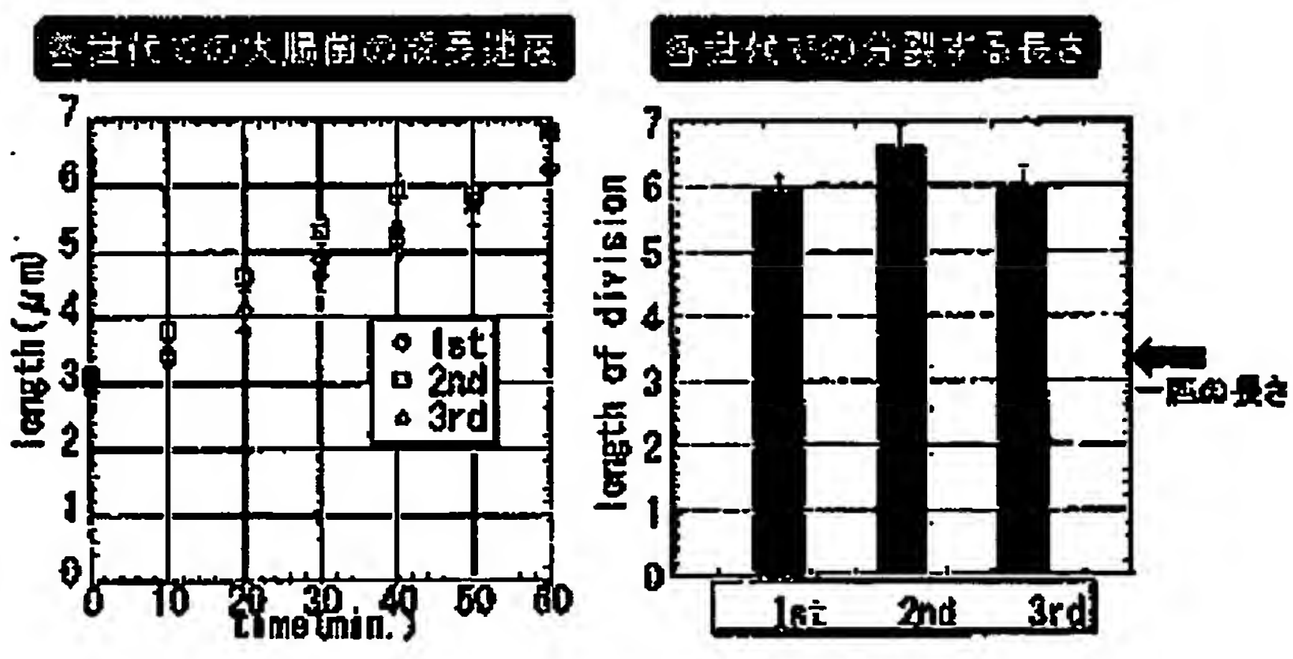
【図１５】



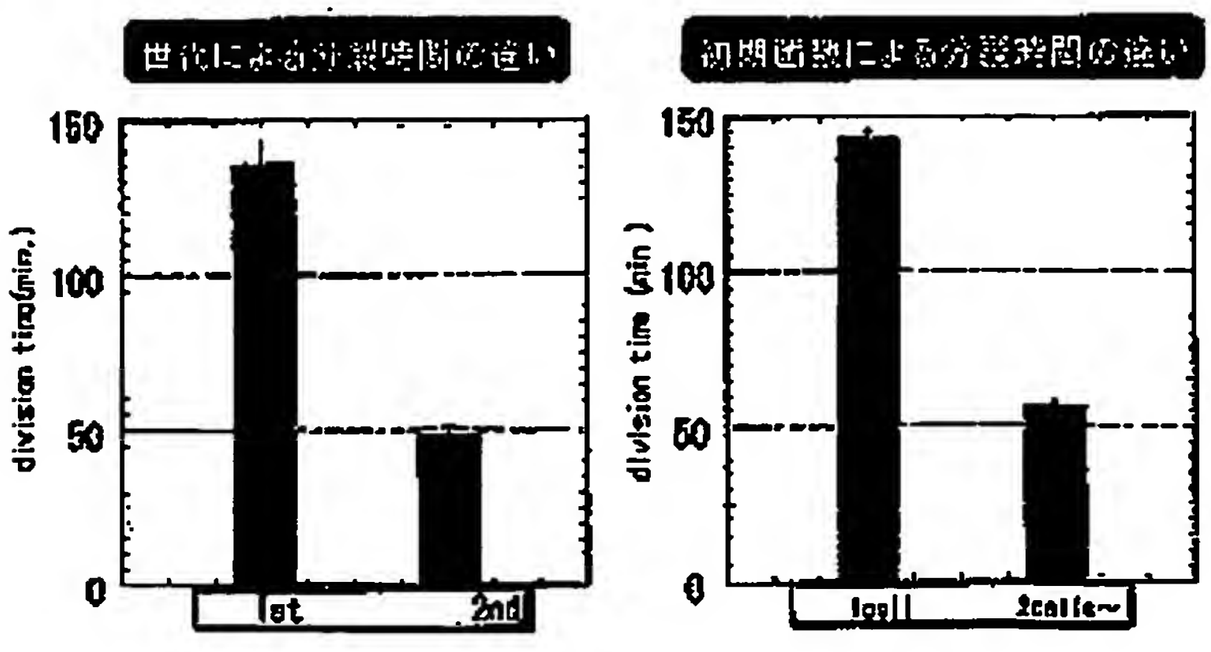
【図１６】



【図１７】



【図１９】



(72)発明者　森口　裕之
東京都世田谷区代田２－36－20　ハイツ浅野101号

Ｆターム(参考)　4B029 AA02 AA07 AA08 AA09 BB11
DD06 DF05 DF06 DG06 FA01
FA04 FA09 GA03 GB02 GB03
GB06 GB08 GB09 HA05 HA10